CONSEQUENCES OF DIRECTED MITOCHONDRIAL DNA DAMAGE IN PRIMARY NEURONS

Matthew John Geden

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Cell Biology and Physiology

Chapel Hill
2017

Approved by:

Mohanish Deshmukh

James Bear

Cyrus Vaziri

Ben Major

Anne Taylor

ABSTRACT

Matthew John Geden: Consequences of directed mitochondrial DNA damage in primary neurons
(Under the direction of Mohanish Deshmukh)

While the consequences of nuclear DNA damage have been well studied in a number of cell types, the exact consequences of acute and targeted mitochondrial DNA damage is less well understood. This dissertation focuses on the examination of the outcomes of DNA damage directed at neuronal mitochondrial DNA in the absence of nuclear DNA damage. I have used a microfluidic chamber model that allows for the spatial and fluidic isolation of neuronal soma (containing the nucleus and mitochondria) from the axons (containing mitochondria). I found that exposure of the DNA-damaging drug cisplatin selectively to the axons was capable of inducing mtDNA damage in the axonal mitochondria without nuclear damage,. Exposure of neuronal axons and mitochondria to the DNA damaging drugs cisplatin, d4T, or camptothecin all resulted in the degeneration of the exposed axons. As there are three well-recognized pathways which are known to regulate axon degeneration in neurons, I investigated their roles in cisplatin-induced axon degeneration. First, I found that deficiency of the proteins Bax and Caspase was unable to save neuronal axons from degeneration. Additionally, inhibition of caspase activity using a pan-caspase inhibitor also was unable to prevent axon degeneration. These findings indicate that the axon degeneration induced by

cisplatin exposure is not mediated by the apoptosis degenerative pathway or the axon pruning pathway. I found that deficiency of Sarm1, an essential component of the Wallerian axon degeneration pathway, was also unable to save against this degeneration. However, addition of the antioxidant glutathione was capable of inhibiting cisplatin-induced axon degeneration. Thus, we find that the axon degeneration induced by cisplatin damage to mtDNA does not appear to be mediated by any of the known pathways of axon degeneration. I also found that cisplatin exposure to axonal mitochondria led to a number of deficits in mitochondrial function—including mitochondrial aggregation, loss of mitochondrial membrane potential, production of reactive oxygen species, and inhibition of mitochondrial trafficking. These findings indicated that directed mtDNA damage, in the absences of nuclear DNA damage, is capable of inducing significant mitochondrial deficits and eventually inducing cellular degenerations.

To my family

ACKNOWLEDGEMENTS

The work I describe here would not have been possible without the help, encouragement, and support of many people. First, I would like to thank my PhD advisor, Mohanish Deshmukh. He has been an incredible source of encouragement to push the boundaries of our lab's research capabilities, to explore new research frontiers fearlessly, to further cultivate critical thinking skills, and to share what it means to be a mentor with me. To all of the members of the Deshmukh lab, thank you so much for your camaraderie, scientific zeal, guidance, and enthusiasm. Coming to work with you every day has been an absolute pleasure and I could not have asked to work with better labmates and scientists at my side. I would like to especially thank Vivian Gama for her mentorship and friendship over the years, you helped me so much through the challenges of graduate school, academic research, and life.

I would also like to thank my thesis committee members—Jim Bear, Ben Major, Anne Taylor, and Cyrus Vaziri—for their encouragement, advice, and scientific points of view.

I do not think that my time in graduate school would have been possible without my incredible friends who were there to lend a hand, an ear, or a laugh when I needed it most. I have learned much about myself from our times together and I will always hold our memories dear.

Finally, I would like to thank my family. Mom and Dad, you have given me the opportunities and the tools to grow and thrive, for your love and support, and for

knowing that you will always be there. I have learned so much from you both and I absolutely could not have asked for better role models and friends in my life. I will always be grateful for everything you have given me. To my grandparents, thank you for all of your incredible hard work and dedication over the years that given us all the opportunities we now enjoy and for making a loving and caring family.

PREFACE

The text in Chapter One of this dissertation, on the pathways of axon degeneration, was previously published. Permission to include the article by Elsevier publishing group:

> Geden MJ, Deshmukh M. 2016. Axon degeneration: context defines distinct pathways. *Curr Opin Neurobiol* **36**: 108-115.

Chapter Two of this dissertation is not yet published.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

8-oxodG: 7,8-dihydro-8-oxo-2’-deoxyguanosine

AP sites: Apurinic/apyrimidinic sites

APP: Amyloid Precursor Protein

ATP: Adenosine triphosphate

BER: Base excision repair

CIPN: Chemotherapy-induced peripheral neuropathy

Casp3: Caspase-3

Casp6: Caspase-6

Casp9: Caspase-9

Cis: Cisplatin

Cyt c: Cytochrome c

DIV: days cultured in vitro

DNA: Deoxyribonucleic acid

DR6: Death Receptor 6

ETC: Electron transport chain

EndoG: Endonuclease G

GSH: Glutathione

GSSG: Glutathione disulfide

IMM: Inner mitochondria membrane

JNK: c-Jun-N-terminal kinases

Kbp: Kilobasepair

LP-BER Long patch base excision repair

NAD+: nicotinamide adenine dinucleotide (oxidized)

NADH: nicotinamide adenine dinucleotide (reduced)

NAM: Nicotinamide

NER: Nucleotide excision repair

NGF: Nerve growth factor

NHEJ: Nonhomologous end-joining

NMNAT1: nicotinamide mononucleotide adenyltransferase 1

OMM: Outer mitochondria membrane

OXPHOS: Oxidative phosphorylation

POLG: mitochondrial DNA polymerase gamma

POLRMT: DNA-directed RNA polymerase, mitochondrial

QVD: Q-VD-OPh hydrate

ROS: Reactive oxygen species

SARM1: Sterile Alpha and TIR Motif 1

SCG: Superior cervical ganglion

SOD: Superoxide dismutase

SP-BER: Short patch base excision repair

TrkA: TrkA

UBE4b: Ubiquitination factor E4B

Wlds: Wallerian degeneration slow mouse

XIAP: X-linked Inhibitor of Apoptosis Protein

XIAP: X-linked Inhibitor of Apoptosis protein

kDa: Kilodalton

mtDNA: Mitchondrial DNA

mtDNA: Mitochondrial DNA

mtSSB: mitochondrial single stranded DNA binding protein

mtSSB: mitochondrial single-stranded DNA binding protein

rRNA: Ribosomal RNA

tRNA: transfer RNA

CHAPTER 1: INTRODUCTION

1.1 The mitochondrion

Mitochondria are highly conserved, double-membrane structured organelles found in almost all eukaryotic cells including all four kingdoms of Eukarya-Plantae, Protista, Animalia, and Fungi. As mitochondria are known to be almost universally conserved across these life forms, and mitochondrial failure has catastrophic consequences for the majority of organisms, mitochondria are broadly recognized as being an essential component of eukaryotic survival and success. Mitochondrial research over the last two centuries has provided significant insights into their origin and function. These now widely-recognized organelles initially were described by their physical morphology in the mid 1800's where they gained their name from combination of the Greek word for threads-"mitos" and the word for grain or granule-"chondros" (1). After their initial discovery, mitochondrial research focused primarily on examining their metabolic functions and contributions to cellular biology such as oxidative phosphorylation and the citric acid cycle (1). Since then, mitochondria have been recognized as serving a number of additional critical functions in cells including cell cycle control (2), organismal development (3), cellular differentiation (4, 5), and cell death (6, 7).

Mitochondrial origins however, are believed to have occurred outside of eukaryotes. The endosymbiotic theory of mitochondrial genesis hypothesizes that

mitochondria were originally prokaryotic cells capable of oxidative respiration which later became incorporated and internalized within a eukaryotic cell as an endosymbiote (8-10). This ancient event is believed to have allowed the host cell the ability to undergo oxidative respiration while propagating the newly internalized primordial mitochondrion. Because mitochondria contain a number of functional elements similar to as bacteria, including mechanisms for replication and transportation of proteins, this is generally thought to be the origin of mitochondria, although other hypotheses exist as well (10, 11).

Mitochondrial structure

These double-membraned organelles are relatively small, ranging from 0.75 to 3 μm in diameter, although the exact morphology and number contained within a single cell tends to vary significantly with cell type (1, 12). One of the most physically distinguishing aspects of mitochondrial biology is that they contain two highly specialized phospholipid bilayer membranes (illustrated in Fig. 1.1). The two membranes involved are commonly referred to as the inner mitochondrial membrane (IMM) and the outer mitochondrial membrane (OMM). The OMM forms a continuous and smooth surface that covers the entirety of the mitochondrion and tends to be somewhat permeable, containing specialized import channels for large polypeptides and permitting diffusion of uncharged proteins under 5,000 kDa (13). As such, small proteins can penetrate through the OMM from the cytosol into the intermembrane space between the IMM and the OMM. In contrast to the OMM, the IMM is highly impermeable and has a distinct, highly folded membrane surface which forms folds

known as cristae (1, 14). The IMM maintains its impermeability through a number of mechanisms, including the absence of porins and the inclusion of specialized lipids (15). The impermeability of the IMM plays a significant role in affecting the ability of the mitochondrion to undergo oxidative phosphorylation. This occurs by engagement of the electron transport chain (ETC) machinery, which exists on the IMM, by allowing the ETC to establish a charge gradient across the IMM in the form of hydrogen cations (16). Embedded between the IMM and the OMM is the transmembrane space which contains a variety of proteins, such as cytochrome *c*, which are essential for metabolisms and cell survival (1, 17, 18). The space contained within the bounds of the IMM is known as the mitochondria matrix. The mitochondrial matrix contains the mitochondrial genome, consisting of a circularized strand of double stranded DNA, as well as key enzymes and metabolites involved in the citric acid cycle, oxidative phosphorylation, and other metabolic pathways (19).

Oxidative phosphorylation and mitochondria energy production

Because over 90% of a cell's ATP comes from the mitochondria, their contribution to cellular metabolism and energy production is hard to understate (20). Within the mitochondria, the majority of energy production comes from the oxidative phosphorylation metabolic pathway. Briefly, oxidative phosphorylation imports pyruvate from cytosolic glycolysis and converts it into acetyl-CoA, *via* pyruvate dehydrogenase, to incorporate into the citric acid cycle. The citric acid cycle takes place within the mitochondrial matrix and cyclically incorporates acetyl-CoA to produce succinate and nicotinamide adenine dinucleotide (NADH). These precursors are then incorporated

into reduction-oxidation (Redox) reactions, using oxygen as an oxidizing agent, and mediated by the ETC to generate an electropotential gradient. The ETC is comprised of five large protein subunits that are imbedded within the IMM. These complexes are known as Complex I (NADH dehydrogenase), Complex II (succinate dehydrogenase), Complex III (cytochrome c reductase), Complex IV (cytochrome c oxidase), and Complex V (ATP synthase) (illustrated in Fig. 1.2) (16, 21). The redox reactions occurring in the ETC passes high-energy electrons derived from NADH and succinate down the ETC from Complex I to Complex IV, which is utilized to actively establish a proton (H^+) charge gradient across the IMM as the ETC pumps hydrogen ions from the mitochondrial matrix into the mitochondrial intermembrane space. This charge gradient establishes the mitochondrial matrix as a highly electronegative region with a strong electrochemical gradient across the IMM. Complex V, also known as ATP synthase, harnesses this electropotential gradient to generate ATP by coupling the lower energy adenosine diphosphate (ADP) to an inorganic phosphate ion (P_i), utilizing the proton gradient for this energetically-demanding step by importing hydrogen ions back into the mitochondrial matrix (22). Thus, ATP synthase directly couples the redox reactions occurring within the ETC and the citric acid cycle to the generation of ATP.

1.2 Mitochondrial DNA and its replication

A brief overview of mitochondrial DNA

Mitochondria contain their own fully separate genome that replicates and exists completely independently from nuclear DNA. The mitochondrial genome is commonly referred to as mitochondrial DNA (mtDNA) and exists in humans as a 16.6 kilobase pair

(kbp) circularized double-stranded DNA genome (23). In mice, the mtDNA is a 16.3 kbp genome (24). The mtDNA encodes for 13 of the 90 essential protein components of the ETC , as well as encoding for all 22 of the mitochondria transfer RNAs (tRNAs), and small (12S, MTRNR1) and large (16S, MTRNR2) ribosomal RNAs (rRNAs) subunits (Fig. 1.3) (23, 25). The 13 peptide coding elements specifically encode seven components of the ETC's Complex I, one component of Complex III, three components of Complex IV, and two components of Complex V (26). The total number of distinct proteins in the mitochondria has been shown to be over 1,500 (27, 28). So while 13 of those proteins are encoded by the mtDNA, the remainder of the components of the ETC and oxidative phosphorylation remains encoded by the nucleus. Indeed, while the genetic material contained on the mtDNA is key for effective mitochondrial function, the remaining proteins that are expressed and localized in the mitochondria are encoded by the nucleus.

Unlike the nucleus, which contains two chromosome sets of genetic material, a single mitochondrion contains between 2 to 10 copies of mtDNA (25, 29, 30). These mtDNA copies exist on the inner surface of the mitochondria matrix as nucleoids which are colocalized with a number of proteins involved in dynamic mitochondria processes such as transcription and replication, including mitochondrial transcription factor A (TFAM) (30-32). Differing again from the nuclear DNA, where one copy of each chromosome is inherited from both a maternal and paternal source, mtDNA is derived soley from the maternal lineage (26, 33). Paternal mtDNA has been observed to be selectively eliminated from sperm mitochondria as part of the spermatogenesis pathway by the mitochondrial nuclease activity (34-37). A minor fraction of paternal sperm

mtDNA appears to persist through this process (~100 copies/sperm) but is believed to be marked *via* a ubiquitination flag for destruction after fertilization, although this process has been observed to fail to occur in abnormal embryos (38-40).

Mitochondrial DNA replication

The replication of mtDNA is an essential process for the maintenance and survival of both the individual cell as well as on an organismal level. Unlike genomic DNA, mtDNA is not replicated only once every cell cycle (41). Instead, mtDNA is believed to be continuously replicated throughout the life of a cell, as mitochondria are known to divide and merge together as a normal biological process (known as mitochondrial fission and fusion, respectively) as well as to be eliminated (41-45). Importantly, mtDNA replicates in post-mitotic tissues (e.g. neurons, cardiomyocytes) as well as in dividing cells (46). As mitochondria replicate and grow, it is critical for mitochondria to retain an appropriate and adequate mtDNA copy number with high fidelity of the replicated genomes. Indeed, altered mtDNA copy numbers have been implicated in a number of diseases including cancer, neurodegeneration, and aging (47-50).

Mitochondria contain their own distinct set of DNA replication machinery which is known as the mitochondrial replisome. This replication machinery is interestingly not encoded with the mtDNA, but is instead encoded by the nucleus (41). Genetic defects in the nuclear DNA encoding these proteins has been implicated in a number of known human diseases (45, 51, 52). The core components of the mitochondrial replisome are the mammalian mitochondrial DNA polymerase gamma (POLG), the mitochondrial DNA

helicase TWINKLE, mitochondrial single-stranded DNA binding protein (mtSSB), and DNA-directed RNA polymerase, mitochondrial (POLRMT) (53). POLG is the only identified mitochondrial DNA polymerase and is actually a holoenzyme comprised of the POLGA catalytic subunit which provides 5'-3' exonuclease activity and the POLGB (also known as P55) catalytic subunit containing DNA processivity activity. TWINKLE appears to play a key role in opening the double stranded mtDNA to allow the unwinding of short double-stranded DNA fragments with a 5'-3' directionality to allow entry of POLG. POLRMT is believed to provide RNA priming activity for POLG to allow for replication and mtSSB is known to tightly bind to single-stranded DNA and is thought to stabilize it. In addition to these key mediators of mtDNA replication, several other proteins are believed to be additionally important for successful long-term propagation of DNA which includes the mitochondrial transcription factor TFAM, the helicase/endonuclease hDNA2, and the helicase hPIF1 (45, 54). While TFAM does not appear to be strictly required for the replication of mtDNA, the genetic depletion of TFAM leads to depletion of mtDNA copy numbers which suggests that it plays an important role in the maintenance of mtDNA (31, 55, 56).

1.3 Mitochondrial DNA damage

Damage to the mtDNA has been well studied and is known to occur in response to a variety of both endogenous and exogenous factors. (41, 57). As mentioned previously, damage to the mitochondrial DNA can lead to number of negative outcomes and is associated with several diseases (26, 45). Potential endogenous mtDNA damaging events include expected events such as mismatch repair, aberrant DNA

replication, hydrolytic decomposition, or exposure to oxidizing ROS generating from oxidative phosphorylation. Potential exogenous sources of mtDNA damage include ultraviolet radiation, ionizing radiation, and genotoxic chemical compounds.

Deletions

Deletions in mtDNA are relatively frequent events that occur natively during mtDNA replication and propagation within somatic cell populations. Depending on the exact deletion event, and to the exact region of mtDNA targetted, the outcome can vary from not significant to toxic. A single deletion event within a single mtDNA copy in a cell will likely be tolerated without incident. However, if significant propagation of the deletion-damaged mtDNA genome occurs it can lead to mitochondrial toxicity and the development of a disease state. Somatic mtDNA deletion events can occur over the lifetime of an organism leading to the accumulation of mtDNA deletions. Indeed, in aging these accumulated mtDNA deletions have been implicated in aging-associated tissue degradation (e.g. neurodegeneration, muscle fiber loss) (58, 59). Somatic mtDNA deletion events accumulated over the lifetime of an organism have also been implicated in cancers (60). The formation of mtDNA deletions within the maternal germline has been implicated in diseases which occur during development. Unlike somatic mtDNA deletion events, these deletions are widely propagated throughout the host and have been indicated in a number of degenerative diseases including progressive external ophthalmoplegia and Kearns-Sayre syndrome (61-63).

Mismatched bases

During normal replication of mtDNA genomes or DNA repair activities, POLG has the potential to incorporate a mismatched nucleotide which leads to a mismatched base. These events are thought to be infrequent, as POLG is a high-fidelity polymerase with an error frequency of ~1 error in every 500,000 bp (64). If mtDNA mismatches do occur they can be repaired by the mismatch repair pathway, but if they fail to be accurately repaired then these mismatch sites can lead to mtDNA mutations.

Oxidative damage

As mitochondria are home to the oxidative phosphorylation and the ETC in the cell, and the mtDNA exists on the inner matrix surface of the IMM which also contains the ETC, it is expected that mtDNA is exposed to a non-trivial amount of oxidative damage (65). Indeed, it has been known that mtDNA is more likely to incur oxidative damage than nuclear DNA as well as being slower to repair the oxidative damage once it occurs (66). The ROS generated within cells is known to be able to induce small oxidative changes that deform nucleotides. Damage can include the generation of an abasic site, oxidized sugar backbones, or oxidized deoxyribose rings (67). The primary product of oxidative damage to pyrimidine bases is considered to be thymine glycol, while the primary product of oxidative damage to purine is likely 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxodG)(57). Thymine gycol tends to be less mutagenic but can inhibit POLG processivity until repaired, while 8-oxodG possess a greater mutagenic potential as 8-oxodG is capable of G:T transversions (57, 68).

Apurinic/apyrimidinic sites

Apurinic/apyrimidinic sites (AP sites), also known as abasic sites, are a form of mtDNA damage where the DNA has completely lost the purine or pyrimidine normally located within the DNA backbone. The formation of AP sites is known to occur in response to spontaneous depurination by hydrolytic deamination of mtDNA bases. The formation of AP sites is also a key step during DNA repair pathways (69).

Strand breaks

Strand breaks in mtDNA can occur as both single-stranded and double-stranded breaks. These breaks can occur in response to both endogenous and exogenous stimuli. Endogenous sources of mtDNA strand break damage include defective replication events or aberrant repair of mtDNA damage. Known exogenous sources of mtDNA strand breaks include ultraviolet radiation, ionizing radiation, environmental toxins, and chemotherapeutic drugs (45). Interestingly, the mitochondrial polymerase POLG is the only human polymerase which is able to effectively incorporate the nucleoside analog drugs used for antiretroviral therapy into the polymerase and the mtDNA (70). The incorporation of these nucleoside analog drugs into the mitochondrial genome leads to the termination of strand synthesis *via* POLG. Abortion of strand synthesis by POLG induced by nucleoside analogs leads to a significant single-strand break event that can later lead to a double-strand break (65). Single-strand breaks alone within the mtDNA backbone are repairable events with the native mtDNA repair pathways. The formation of a double-stranded break to the mtDNA is more challenging to repair and can lead to more significant damage to the mtDNA (57).

Alkylation and adducts

The sources of alkylating damage to the mitochondrial DNA primarily comes from exogenous sources, although endogenous sources of alkylating damage have been identified (e.g. S-adenosylmethionine)(71, 72). A number of genotoxic agents such as tobacco smoke, environmental toxins, and chemotherapeutic agents have been identified to bind and form adducts with mtDNA (45, 65). Specifically, the platinum class of chemotherapeutic drugs, which contains cisplatin, is known to bind DNA and form a variety of adducts including single-base adducts as well as both inter- and intra-strand crosslinks, typically between guanine bases (73). In the case of cisplatin the most prevalent damage induced is the intrastrand crosslink of 1,2-d(GpG), with others occurring at a significantly reduced frequency (65, 74). Intrastrand crosslinking by bulky adducts such as cisplatin can have catastrophic consequences on the mtDNA. As intrastrand crosslinking events prevent the mtDNA from opening and inhibit both transcriptional and translational machinery, this functionally renders a stretch of the mtDNA unusable. In addition, any attempt by this machinery to process through the damaged site can lead to strand breakage of the mtDNA proximal to the site of damage (75).

1.4 The repair of mitochondrial DNA

Like the nucleus, mitochondria contain DNA repair pathways capable of addressing DNA damage that may occur the mtDNA. These DNA pathways have a number of parallels to nuclear DNA repair pathways, but remain distinct.

Base excision repair

Base excision repair (BER) is considered to be the primary pathway engaged for the repair of the bulk of mtDNA damage. Indeed, BER is the best characterized mtDNA repair pathway and is engaged to repair the most common forms of oxidative damage, non-helical distorting lesions, AP sites, single-strand DNA breaks, and some types of alkylating damage (76). As mtDNA does experience a higher rate of oxidative damage, it is interesting to note that mitochondrial BER is actually more proficient at repairing the most common type of oxidative DNA damage (8-oxy-dG) than in the nucleus (77). There are two subtypes of BER known to mediate repair, short patch BER (SP-BER) and long patch BER (LP-BER). SP-BER is used to replace single bases and is the primary type of BER implicated in mitochondria, although the machinery required for LP-BER has been reported to be found in the mitochondria (Fig. 1.4) (78, 79).

Mitochondrial BER is mediated *via* a series of well-defined stepwise processes that lead to the successful repair of damaged mtDNA. Specifically, these steps include the recognition of the damage site, excision of the damaged base to generate an AP site, DNA strand cleavage at the AP site, strand end processing, strand gap filling, and ligation of the repaired strand (57). Base recognition and excision in mitochondrial BER occurs *via* DNA glycosylases, which cleave the N-glyosylic bond between the damaged base and the DNA backbone to form an AP site. Mitochondria contains four bifunctional DNA glycosylases (OGG1, NTHL1, NEIL1, and NEIL2), which can recognize either damaged ssDNA or dsDNA, and two monofunctional DNA glyosylases (UNG1 and MUTYH), which only recognize dsDNA damage sites (76). Mitochondrial APE1 then

processes the ends at the cleavage site, POLG fills in the gap, and then the ends are sealed and ligated by DNA Ligase IIIα.

Nucleotide Excision Repair (NER)

NER is a robust and distinct pathway for the repair of single-stranded DNA and is capable of repairing damage that induces significant conformational change to the DNA helix. It has been largely implicated in the repair of large bulky adducts such as cisplatin-induce intrastrand-crosslinks, damage caused by intercalating agents, radiation-induced adducts, and those produced by environmental toxins. These damage events, which are not readily repaired by the BER pathway, are repaired by NER-mediated DNA repair (80). This pathway of DNA repair is utilized by the nucleus for maintenance and repair of nuclear DNA. However, there is no evidence for the involvement of the NER repair pathway in mammalian mitochondria (81, 82). Thus, certain types of DNA damage that normally require NER for repair in the nucleus (such as cisplatin intrastrand crosslinks) do not appear to be repairable within the mitochondria (45, 57, 80, 82-84). Importantly, these damage types are therefore more likely to be capable of inducing more significant and long term consequences when they induce damage to mtDNA than to nuclear DNA.

Mismatch repair

Mismatch repair has been well reported as an important and conserved mediator of the removal and replacement of mismatched nucleotide incorporation into the nuclear genome. In mammalian mitochondria however, this process has not been as clearly

implicated as an essential process for the maintenance of the mitochondrial genome (85). One report has described the existence of a mismatch repair-like system in rat mitochondria, but this process does not appear to have a clear strand preference for repair of the mismatched base (86). Additionally, this process did not appear to be dependent on the reported nuclear mismatch repair components (MSH family proteins, homologs of bacterial MutS) (86). More recent research has indicated the repair factor YB-1 may be a mitochondrially relevant mediator of mismatch repair, but its exact contribution remains unclear (87).

Double-strand break repair

The repair of double-stranded breaks in the nucleus is primarily achieved through either homologous recombination or by nonhomologous end-joining (NHEJ). There is evidence that yeast and plants are capable of repairing mtDNA double-strand breaks by homologous recombination pathways, however homologous recombination does not appear to occur in significantly detectable levels in mammalian mitochondria (88-91). Thus in mammalian mitochondria, homologous recombination is not a major pathway engaged in the repair of double-stranded DNA breaks. NHEJ represents the other potential pathways for the repair of double-strand breaks in mtDNA. Indeed, extracts of mitochondria have been found to be capable of joining together broken double-stranded DNA *in vitro (92)*. However, this process occurs at low frequencies and is not thought to be a major contributor to the repair of double-stranded breaks in mammalian mtDNA (57, 92). Thus, mitochondria do not appear to be highly competent in the repair of double-stranded DNA breaks, although this area still requires additional study.

1.5 Mitophagy

Damaged or aberrant mitochondria can have negative consequences for a cell and an organism. These damaged mitochondria are capable of producing damaging amounts of ROS, consuming cellular resources, and potentially inducing apoptosis. As such, cells have developed a mechanism that permits the selective elimination of these damaged mitochondria known as mitophagy (93). Mitophagy is a specialized form of cellular autophagy that leads to the flagging of damaged mitochondria to be degraded utilizing a subset of the components of the autophagy pathway.

Damaged mitochondria are recognized by the mitophagy pathways by loss of the mitochondrial membrane potential across the IMM (93). Deterioration of the ability to maintain a sufficient electrochemical membrane potential gradient across the IMM often results from a critical failure of the ETC and oxidative phosphorylation. Failure of these pathways suggests that the mitochondrion is unfit to maintain its basic functions and should be eliminated before it can induce damage to the cell as a whole. The loss of membrane potential is known to lead to the stabilization of interaction of the PINK1 protein and the OMM (94-96). In healthy mitochondria, PINK1 undergoes import through the OMM and is locally degraded within the transmembrane space, thus inhibiting a stable association with the OMM (94, 95). Once stabilized, PINK1 phosphorylates a number of OMM targets including S65 of OMM ubiquitin residues (97). The ubiquitin ligase Parkin is recruited from the cytosol to the OMM, as Parkin has a high affinity for this modification. Once associated with the OMM of a depolarized mitchondria, Parkin undergoes a conformational shift and becomes capable of executing the addition of polyubiquitination chains to the OMM . PINK1/Parkin activity

leads to the conjugation of a number of different types of polyubiquitin chains to the OMM (including K48-, K63-, K11-, and K6-linked chains) (98). This polyubitination of the OMM leads to the initiation of the formation of an isolating double-membraned autophagosome around the flagged mitochondrion, which is mediated by a number of now well-defined autophagy-associated proteins (98, 99). Once fully engulfed by the autophagosomal membrane, the mitochondrion undergoes complete degradation upon the fusion of the autophagosomal membrane with a lysosome (93).

1.6 Mitochondrial ROS

Mitochondria are a key source of ROS within mammalian cells. Specifically, superoxide radical is the predominant form of ROS generated by the mitochondria (100). Superoxide is generated in the mitochondria as a consequence of oxidative phosphorylation and the activity of the ETC, where Complex I and Complex III are major contributors (100). The superoxide free radical is managed by the mitochondria by constitutively degrading it into less reactive forms, such as the conversion of superoxide into H_2O_2 by mitochondrial superoxide dismutase (SOD) (101). H_2O_2 is later degraded and converted into water by the activity of glutathione peroxidase and peroxiredoxins (102). The degradation of H_2O_2 is an important step, as if left unchecked it can lead to the generation of hydroxyl radicals though an interaction with Fe^{2+}, known as the Fenton reaction (100, 102). The hydroxyl free radical is highly reactive with almost all biomolecules and can lead to significant damage to cellular components (e.g. lipids, proteins, nucleic acids) (100, 102). Superoxide and various other forms of ROS molecules are now thought to play a number of important roles in cellular signaling

pathways important for proper cellular homeostasis (103, 104). Although ROS signaling is utilized for a number of cellular functions, mitochondria production of ROS leads to higher levels of localized ROS within the mitochondria. Indeed, the mitochondrial matrix under basal conditions is known to contain between 5- to 10-time the relative amounts of superoxide found within the cytoplasm or nucleus (102). Damage to the mtDNA is known to lead to dysfunction of oxidative respiration in mitochondria and the aberrant and excessive formation of damaging ROS species (105, 106).

Glutathione (GSH) is a key endogenous antioxidant produced by the cell which helps mediate the consequences of ROS, including by reducing and eliminating various ROS species and free radical ions (107). GSH functions as a key component of the conversion of H_2O_2 into water in association with glutathione peroxidase (102). In addition to that role, GSH exists throughout both the cell and the mitochondria, providing a broad capability of combating ROS throughout the cell. Upon interaction with an oxidizing agent, reduced cellular GSH is converted into an oxidized state and forms into glutathione disulfide (GSSG). GSSG is then reduced back into reduced GSH by the activity of glutathione reductase (107, 108).

1.7 Mitochondrial Dynamics

Mitochondria exist in a wide variety of morphologies, dependent upon cell type. They exist in dynamic networks, that can range from highly divided and granular to largely fused long threads winding within a cell. They are dynamic and mobile organelles that do not statically exist in a single location within a cell but instead undergo active transport throughout the cell.

Mitochondrial fission and fusion

Early reports of mitochondria described them under static imaging conditions, but upon study in live cells it was discovered that mitochondria are actually capable of undergoing division and fusion (109-111). These division and fusion events of mitochondrial have become known as mitochondrial fission and fusion, respectively. In recent years, there has been a growing appreciation for mitochondrial fission and fusion and their contributions towards mitochondrial stability, health, and responses towards cellular stress. It has been proposed that the fusion of mitochondria may help mediate mitochondria stress response by the fusion of a damaged mitochondria with an undamaged one as a form of complementation to rescue and regenerate the damaged mitochondrion (44). While fusion has been indicated to have roles in mitochondrial repair and maintenance, mitochondrial fission has been instead implicated in the birth and expansion of mitochondria. As damaged mitochondria are eliminated or a cell divides, mitochondria replicate and undergo fission to expand and replenish the pool of healthy and functional mitochondria. Together, mitochondrial fission and fusion has been proposed as a potential mechanism of mitochondrial quality control, cellular growth, responses to changes in cellular metabolism, cell survival and death, diseases, and a number of other key cellular roles (44, 110, 112, 113).

These pathways have become reasonably well understood in mammals in recent years, as their mechanisms appear to be conserved between yeast, flies, and mammals (114). Briefly, the major components of these pathways appear to be members of the dynamin family of large GTPase. The mammalian protein Drp1 is the primary regulators of mitochondrial fission, although other Drp1 binding partners play a role (44,

114). Fusion of mitochondrial instead require alternate dynamin family members, specifically the mitofusins (MFN1 and MFN2) and OPA1. MFN1 and MFN2 are essential mediators of the fusion of the OMM of mitochondria. Interestingly, MFN2-mediated fusion events appear to involve the pro-apoptotic protein Bax (115). Fusion of the IMM is instead mediated by OPA1 and not the mitofusins (114).

Mitochondrial movement

In addition to the dynamics provided by the ability to undergo fission and fusion, mitochondria are also highly mobile organelles. Mitochondria are known to be actively trafficked along the microtubule-based cytoskeleton within a cell. The ability to mobilize and transport mitochondria within the cell allows cells to localize mitochondria to discrete subcellular localizations where they may be required most. One cell type where this is most notable is in neurons. Neurons contain both a cell body and long axons, that can reach distances of up to one meter in humans (116). As such, the ability to successfully transport mitochondria over long ranges is essential for the proper health and survival of an organism. Mitochondria are trafficked through active transportation on microtubules and are powered by microtubule motor proteins-dynein and kinesins (117, 118). Mitochondrial are complexed to these microtubule motor proteins by the mitochondrial cargo adaptor proteins, Miro and Milton (Fig. 1.5) (119, 120). Neurons rely upon these transportation pathways to ensure that all lengths of the neuronal axon are supplied with healthy mitochondria to enable full functionality of the cell across its length (120). Interestingly, the transport of mitochondria appears to be integrated into the mitophagy-based checkpoint control system of mitochondrial

function. If mitochondria become damaged and depolarized, they recruit PINK1/Parkin to them which leads to the interaction of PINK1/Parkin with the microtubule motor tether proteins leading to phosphorylation and subsequent ubiquitin-mediated degradation (120, 121). The loss of Miro means that damaged and defective mitochondria stall out on microtubules and do not undergo trafficking until they are repaired or eliminated.

1.8 Chemotherapy-induced peripheral neuropathy (CIPN)

Chemotherapy-induced peripheral neuropathy (CIPN), a condition in which the peripheral nervous system is damaged by the treatment of chemotherapeutic drugs, can induce significant axon damage causing numerous neurological deficits in cancer patients (122-124). These deficits can initially manifest in a number of ways, leading to symptoms in the hands and feet such as pain, numbness, tingling, as well as sensory loss. These symptoms can progressively worsen throughout treatment, resulting in the development of significant sensory pain, motor ataxia, pain, extreme numbness and loss of sensation (125). Those symptoms make CIPN often the dose-limiting factor in the dosage of chemotherapeutic drugs and reducing the maximum benefit available to patients (126). Among the frontline drugs that are associated with severe CIPN are the platinum compounds such as cisplatin (122-124, 127, 128). Incidentally, peripheral neuropathy is also observed in HIV patients that are prescribed anti-retroviral drugs such as d4T (129). Importantly, while the antineoplastic mechanisms of these drugs are well-studied in proliferating cells (*e.g.* by targeting mitosis and DNA replication pathways), the fact that many of these drugs induce axon degeneration in postmitotic

neurons is mostly unexpected and the exact mechanisms involved remain largely unknown.

The exact pathologies involved in CIPN may vary, but most seem to result in axonal damage which leads to the “dying back” of the axon (130). The “dying back” phenotype is generally regarded as the degeneration of the distal tip of the axon back to the neuronal cell body. Different chemotherapeutic agents are thought to induce CIPN through different mechanisms. For example, chemotherapeutic drugs that disrupt microtubule stability (such as taxanes or vincristine) are known to induce CIPN in peripheral sensory and motor neurons, as these neurons rely upon properly working microtubule networks in their peripheral axons for maintenance of basic functions (131, 132). Other chemotherapeutic drugs, such as the platinum agents, including cisplatin and carboplatin, are DNA damaging agents and it is less clear what the mechanisms are which lead to CIPN in response to platinum therapies.

Cisplatin-induced CIPN

The platinum class of chemotherapeutic agents, including cisplatin, is known to induce their effects by damaging DNA by forming a platinum-DNA adduct. Platinum drugs are known to be relatively severe inducers of CIPN in patients, with up to 90% of patients experiencing sensory neuropathies (133, 134). The neuropathic damage induced by cisplatin is dose-dependent and can lead to long term neurological deficits (130). Cisplatin damages DNA primarily in the form of inter- and intra-strand crosslinks, typically between guanine bases (73). Specifically, the primary form of DNA damage induced by cisplatin is the intrastrand crosslink of 1,2-d(GpG) (65, 74). The CIPN

observed in response to cisplatin has been reported to be capable of inducing the dying back axon degeneration pathology prior to the full loss of the neuronal cell body (130). Some research has shown that in peripheral neurons cisplatin causes both damage to the nucleus as a well as the mitochondria (135-137). However, the exact contribution of this damage and how it leads to the dying back phenotype of axon degeneration prior to neuronal degeneration remains unclear.

1.9 The known pathways of axon degeneration

Neuronal development and target innervation by axons and dendrites are essential processes required for the establishment of the nervous system. Simultaneously however, the selective destruction of neurons or only their projections is also important for the refinement of the wired brain (138). Although, the mechanisms regulating the survival and elimination of neurons *via* apoptosis during development are well studied, the pathways governing the selective degeneration of the axons are less understood. Axon degeneration is predominantly studied in three contexts: 1) Axotomy (also known as Wallerian degeneration) where the severing of axons results in the degeneration of axons distal to the cut site; 2) Apoptosis-induced axon degeneration, where the entire neuron is exposed to apoptotic stimuli (*e.g.* global deprivation of trophic factors) resulting in the degeneration of both axons and soma; and 3) Pruning-induced axon degeneration, which here is referred to here as “Axon Pruning”, where a subset of axons are selectively exposed to pruning stimuli (*e.g.* axon-only or “local” deprivation of trophic factors) which results in the selective degeneration of only the axons exposed to the stimulus (Illustrated in Figure 1.6).

Wallerian degeneration

Wallerian degeneration is a unique and structured form of axon degeneration that occurs when a neuronal axon is crushed or severed (axotomized) from its cell body. Wallerian degeneration has been well examined in the Wld^S mouse, where axotomy-induced axon degeneration is strikingly inhibited (139). Wld^S mice express a unique gene product responsible for this protection which was identified as the fusion of the N-terminal fragment of ubiquitination factor E4B (UBE4b) with NMNAT1 (nicotinamide mononucleotide adenyltransferase 1) (140). These findings implicated the role of NAD+ (nicotinamide adenine dinucleotide) metabolism in Wallerian degeneration, as NMNATs are key [i]enzymes in the NAD+ salvage pathway. NAM (Nicotinamide), which is a byproduct of NAD+ metabolism, is recycled into NMN (nicotinamide mononucleotide). NAD+ can then be regenerated from NMN by the NMNAT enzymes to maintain steady-state pools of intracellular NAD+. The rapid depletion of NAD+ has been identified to be a central event in axons undergoing Wallerian degeneration and addition of exogenous NAD+ (albeit at high concentrations) is sufficient to inhibit this degeneration (141-143). However, recent evidence suggest that rather than NAD+ depletion, it could be the accumulation of NMN instead that triggers axon degeneration (144).

Recently, SARM1 (Sterile Alpha and TIR Motif 1) was identified as an essential mediator of the axotomy-induced axon degeneration (145, 146). SARM1-deficiency robustly protects axons following axotomy *in vivo* and *in vitro* (145, 146) and maintains NAD+ levels *in vitro* (147). Conversely, direct activation of SARM1 rapidly depletes intracellular NAD+ and is sufficient to induce axon degeneration (146, 147). A key event that occurs after axotomy is the depletion of NMNAT2, which is sufficient to

trigger axon degeneration (148). SARM1 appears to be the effector of NMNAT2-depletion induced axon degeneration, as the degeneration trigged by NMNAT2 depletion can be fully rescued by the co-deletion of SARM1 (149). Downstream of SARM1, a MAP Kinase signaling pathway is activated to propagate the degenerative signal (150, 151). This cascade includes DLK (Dual Leucine Zipper Kinase), MEKK4, and MLK2 which leads to the activation of c-Jun-N-terminal kinases (JNKs) *via* MKK4 and MKK7 (150). Exactly how this signaling pathway induces axon degeneration is not fully understood, but Ca^{2+} signaling and calpain activation appear to be important for mediating late events in axon degeneration (152-154).

Apoptosis (apoptosis-induced axon degeneration)

Neuronal apoptosis, which results in the degeneration of both the soma and axons, occurs extensively during development (155). This process has been well studied in the developing peripheral nervous system where sympathetic and sensory neurons are acutely dependent on target-derived nerve growth factor (NGF) and if deprived of NGF will undergo apoptosis (156, 157). This phenomenon can also be recapitulated in cell culture where dissociated sympathetic or sensory neurons, or ganglia explants, are maintained in the presence of NGF. Elimination of NGF from the culture media, to deprive the entire neuron of NGF (often referred to as “global deprivation”), results in the apoptotic degeneration of both the soma and axons (156, 158).

Global deprivation of NGF initiates a well-defined apoptotic pathway that we briefly outline here. NGF deprivation induces the dephosphorylation of its receptor,

Tropomysin receptor kinase A (TrkA), which leads to the activation of a DLK-mediated apoptotic signal. DLK signaling activates downstream mediators (including JNKs, MAPKs, and GSK3β and leads to the transcriptional upregulation of proapoptotic BH3-only family proteins (including Bim, Puma, Bmf, Hrk/DP5) by transcription factors such as c-Jun, NF-Y, and FOXOs (156, 159). Recently, Puma was shown to be a key mediator of axon apoptosis. Not only is Puma induced in the cell body after global NGF deprivation, but Puma deficiency markedly reduces axon apoptosis (159). A major function of the BH3-only proteins such as Puma is to activate Bax, a pro-apoptotic member of the Bcl-2 family, which is required for neuronal apoptosis (156). Some BH3-only proteins directly interact with Bax to induce its conformation activation, while others inhibit anti-apoptotic proteins of the Bcl-2 protein family (Bcl-2, Bcl-w, Bcl-XL, Mcl-1) (160). In particular, Bcl-w and Bcl-XL are known to be localized to axons where they are important for maintaining axonal survival (159, 161).

Once Bax is activated, it translocates to mitochondria and inserts into the outer mitochondrial membrane, leading to the release of cytochrome *c* (cyt *c*) into the cytoplasm (162). Released cyt *c* can then bind to Apaf-1 (Apoptotic protease activating factor 1) to induce a conformational change in Apaf-1 that results in the recruitment of and activation of procaspase-9 (Casp9). Activated Casp9 functions to activate Caspase-3 (Casp3), and subsequently calpains, which together execute the degeneration of both soma and axons by targeting many cellular proteins for proteolysis (153). Interestingly, while there are numerous mammalian caspases with diverse cellular functions, only Casp9 and Casp3 appear to be strictly required for global NGF-deprivation induced neuronal apoptosis (163). Lastly, X-linked Inhibitor of Apoptosis

Protein (XIAP) inhibits caspase activity in both soma and axons, but is degraded during global NGF deprivation (164, 165). Consistent with this, XIAP-deficient neurons exhibit enhanced axon degradation when globally deprived of NGF (165).

This apoptotic pathway activated upon global deprivation of NGF has been recognized to be important for the degeneration of both the cell bodies and axons. Indeed, deletion or depletion of the key effectors of this apoptotic program (*e.g.* DLK, JNKs, c-Jun, FOXO3a, Bax, Apaf-1, Casp9, Casp3) blocks the death of neurons in response to global NGF deprivation (156, 159, 163, 166). Overall, these data suggest that in the context of global apoptotic stimulation, axonal degeneration is a consequence of the apoptotic program activated in the cell body.

Axon pruning (pruning-induced axon degeneration)

In addition to neuronal apoptosis, the nervous system undergoes substantial refinement by the pruning of axons and dendritic connections, which are selectively dismantled and rewired. Pruning is critical not only for establishing specific and appropriate neuronal circuitry during development but also for neuronal plasticity in the adult nervous system (138, 167). Precise spatial and temporal control of the degenerative machinery is important for pruning to ensure that only the targeted regions of the axons are degraded. This spatial regulation is in contrast to apoptosis, where the entire neuron undergoes degeneration.

Similarly to axon apoptosis, mechanistic insights into the axon pruning pathway have primarily come from studies in peripheral neurons (sensory and sympathetic) subjected to NGF deprivation. An important distinction however is that while axonal

apoptosis is triggered by “global” deprivation of NGF (where neurons are completely deprived of NGF), axon pruning is observed in response to the selective deprivation of NGF from the axon while maintaining the neuronal cell body in NGF. To study this pathway *in vitro*, neurons are cultured in compartmentalized chambers (*e.g.* Campenot (168) or microfluidic chambers (169)) where the cell bodies and the axons are maintained in different compartments, separated by a physical barrier, to keep them spatially and fluidically isolated. Selective deprivation of NGF from only the axon compartment (known as “local” NGF deprivation), with cell bodies maintained in NGF, triggers the selective degeneration (pruning) of only the axons in the NGF-deprived axon compartment (168). Although axon apoptosis and axon pruning are both induced by NGF deprivation, the distinction between global and local NGF deprivation is important as it defines the context, and thus, the specific pathway activated.

While multiple factors are known to mediate axon pruning *in vivo* (138), the molecular pathway of axon pruning has been best studied *in vitro* in the context of local (axon-only) NGF deprivation. Amyloid Precursor Protein (APP) and Death Receptor 6 (DR6) have been identified as key regulators of pruning *in vivo* (170, 171). However, loss of either APP or DR6 was not protective against apoptosis (global NGF deprivation) *in vitro* (171), suggesting that APP and DR6 play a unique role in axon pruning but are dispensable for apoptosis. During local NGF deprivation-mediated pruning, the loss of TrkA activity leads to the activation of DLK to induce a retrograde signal to the soma (159). This signal is important, as DLK-deficient neurons are protected from axon pruning (159, 166). DLK activation leads to the local phosphorylation of JNKs to propagate the pruning signal (166, 172). Interestingly, inhibition of JNK (and also p38

MAPK) in only the axons, and not the cell bodies, is sufficient to protect against axon pruning, confirming their roles in transduction of the retrograde signal (172). Furthermore, GSK3 (both α and β) is a potential JNK-regulated mediator of this retrograde signal as inhibition of GSK3 activity in the soma, but not the axons, protects against axon pruning (172). While GSK3 could be activated locally in axons, its kinase activity may be required only in the soma where its substrates important for pruning could be spatially localized. c-Jun has been identified as another soma-dependent transcriptional regulator that is activated during pruning (166, 173). Whether c-Jun is required for axon pruning is undetermined however, as previous results examined the requirement of c-Jun in the context of axon apoptosis (global NGF deprivation) (159, 166).

Recently, the BH3-only protein Puma was identified as an essential mediator for axon pruning where its deficiency protects axons against local NGF deprivation (159). As Bax is also required for axon pruning (170, 174, 175), the role of Puma is likely to directly activate Bax. Consistent with Bax activation, cyt *c* is released from mitochondria in axons undergoing pruning (174), but whether cyt *c* is required for pruning is not known. This is particularly relevant as cyt *c* is known to activate Casp9 *via* Apaf-1 on the apoptosome. However, Apaf-1 is not required for axon pruning (174). While Apaf-1-deficient neurons are protected from undergoing apoptosis after global NGF deprivation (159, 163), they still undergo pruning after local NGF deprivation (174). Interestingly, despite not requiring Apaf-1, pruning requires both Casp9 (174) and Casp3 (174, 176). Thus, Casp9 appears to be activated *via* an Apaf-1-independent mechanism during pruning. This is particularly relevant in post-developmental mature

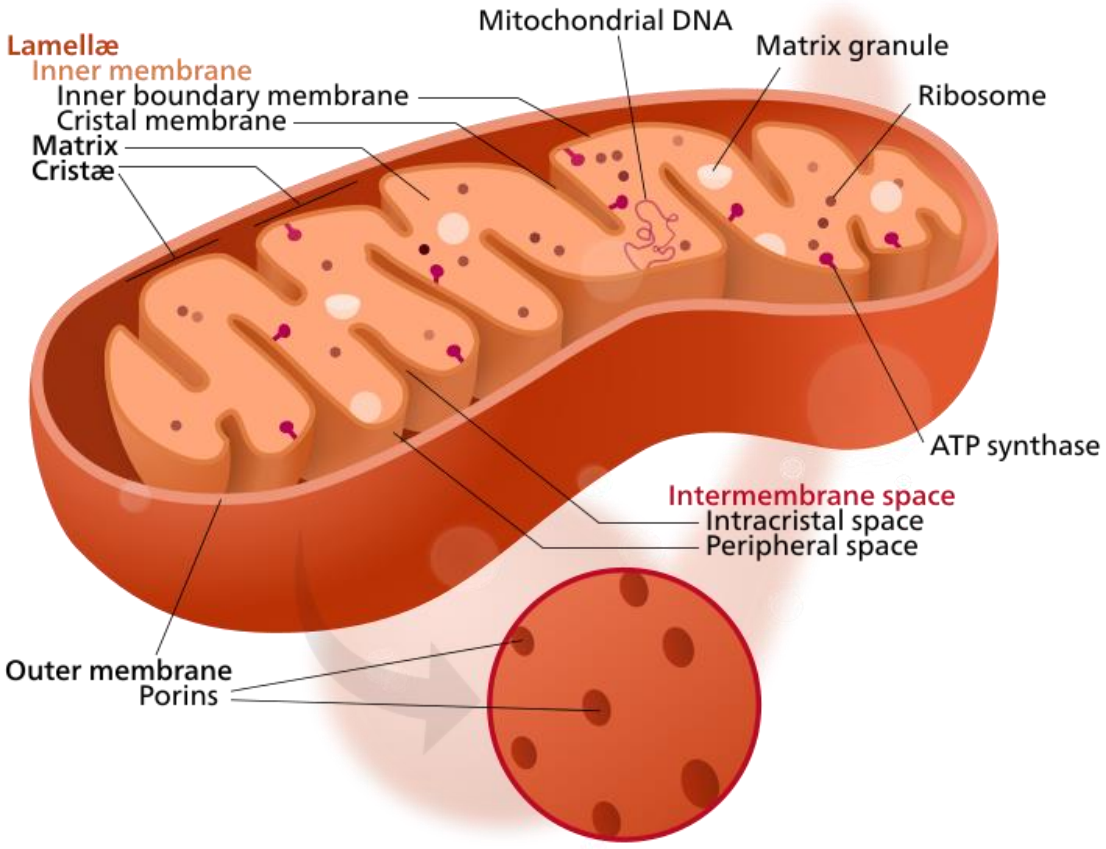
neurons, which are known to shut down the apoptotic pathway by turning off Apaf-1 expression (177), yet maintain plasticity and remain competent to undergo pruning (174). This finding not only supports but highlights the Apaf-1-independent nature of the pruning pathway and illustrates how neurons are able to exert precise and differential control over the apoptosis and pruning pathways. Additionally, pruning is dependent on Casp6, a caspase which is not essential for apoptosis (170, 174, 176). However, exactly how caspases are activated during pruning, or even their precise order of activation, remains unknown. As pruning allows for the selective degeneration only the targeted axon, without degeneration outside of that target region, it is likely that caspase activation during pruning is spatially restricted (178). One such mediator that restricts caspase activity during pruning is XIAP (dIAP in *Drosophila*) (174, 179), which could serve to spatially constrain degeneration to the desired region. Indeed, XIAP-deficient neurons are unable to spatially restrict caspase activity and inappropriately accumulate active Casp3 in their cell bodies during pruning (174).

1.10 Figures and Legends

Figure 1.1: Mitochondrial morphology and macrostructural elements

Mitochondria exist as double membraned organelles with the outer mitochondrial membrane existing a smooth surface enveloping the entire mitochondrion. Within the outer mitochondrial membrane there exists the highly folded inner mitochondrial membrane, the highly folded regions of this membrane are known as the cristae. Between the outer and inner mitochondrial membrane lies the transmembrane space. Within the inner mitochondrial membrane is the mitochondrial matrix, where the mitochondrial DNA exists.

Figure 1.1



(Figure obtained from Wikipedia under the Creative Commons license)

Figure 1.2: The mammalian electron transport chain

Depicted here is the flow of high energy electrion through the mitochondrial electron transport chain. The transfer of electrons produces a lot of energy, which is used to pump protons out of the mitochondrial matrix across the IMM to the transmembrane space. Complex I (I) takes in the initial event and passes the electron down the line with Complex II (II) to Complex (III). Complex III then passes it to Complex IV (IV). At each one of these steps, protons (H+) are pumped across the IMM to produce a mitochondrial membrane potential. The membrane potential is harness by Complex V/ATP synthase to allow proton from across the IMM to produce ATP.

Figure 1.2

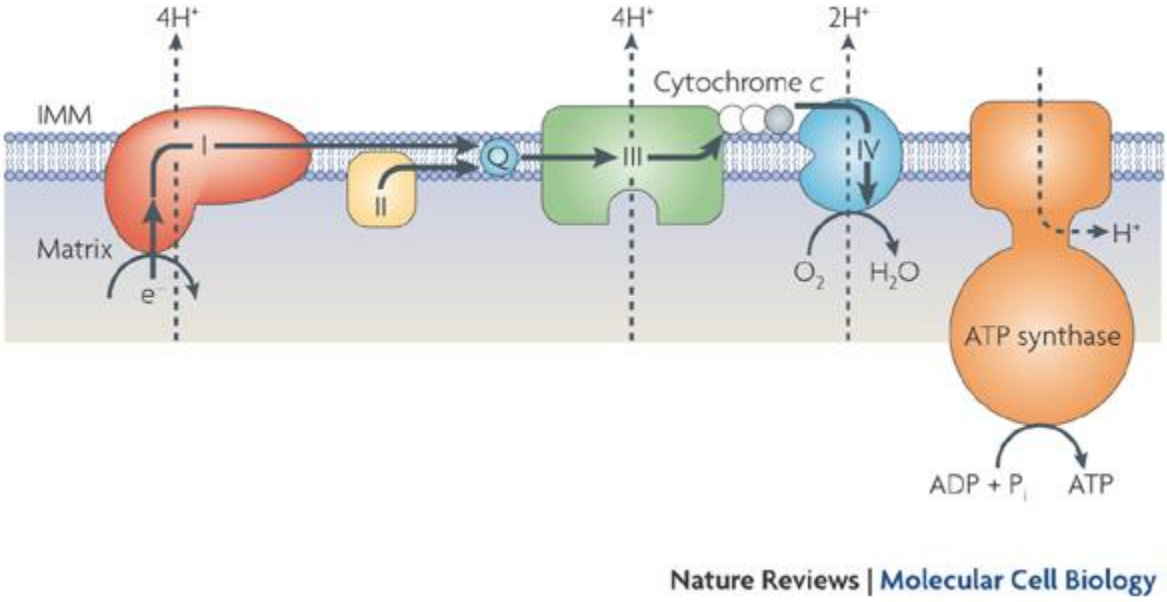
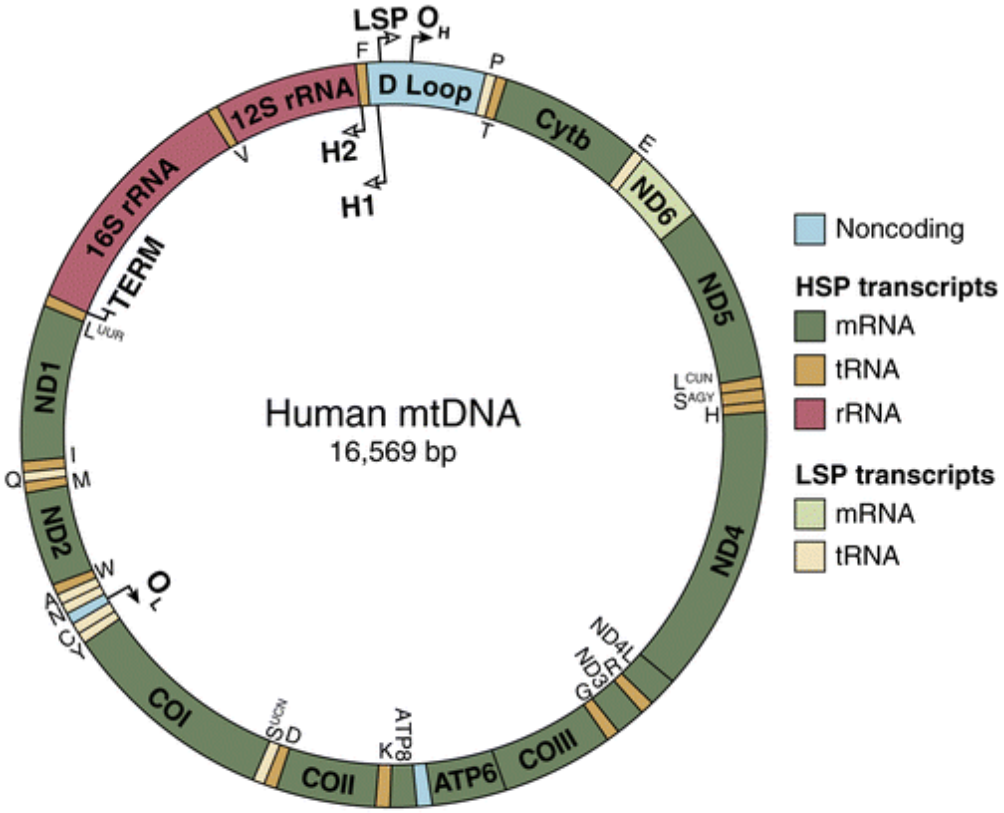


Figure adapted from Ow WP et al, *Nat. Rev. Cell Biol.* (2008)

Figure 1.3: Illustration of human mtDNA

Human mtDNA is a double-stranded, circularized 16,569 base pair genome that exists within the mitochondrial matrix. The mtDNA encodes for 13 of the 90 essential protein components of the ETC , as well as encoding for all 22 of the mitochondria transfer RNAs (tRNAs), and small (12S, MTRNR1) and large (16S, MTRNR2) ribosomal RNAs (rRNAs) subunits.

Figure 1.3

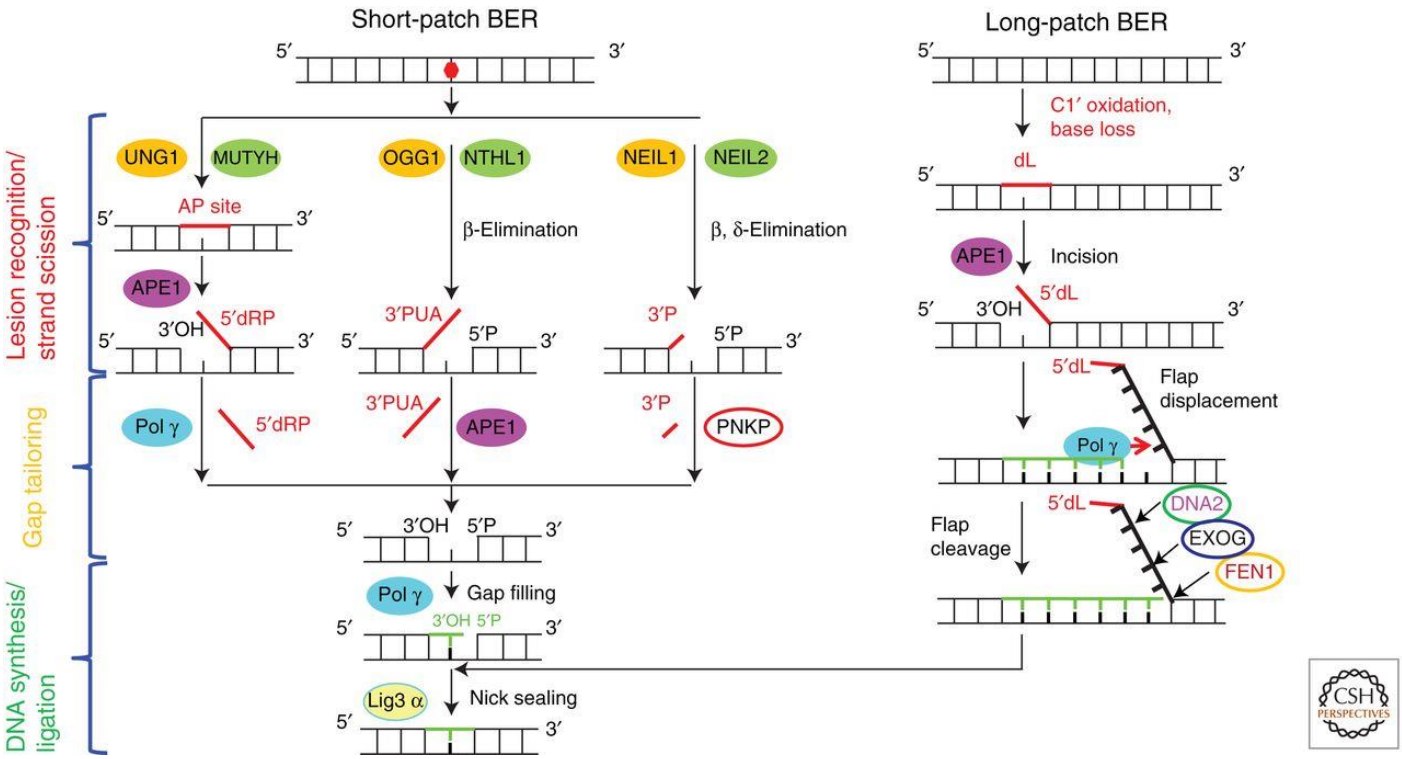


Falkenberg M, et al. 2007.
Annu. Rev. Biochem. 76:679–99

Figure 1.4: The mammalian BER pathway.

Illustrated here is short patch and long patch BER. SP-BER is the predominant form of BER utilized by mitochondria. Mediators of each step are indicated as appropriate. For additional information see Alexeyev M et al. *Cold Spring Harb. Perspect. Biol.* (2013).

Fig. 1.4



From Alexeyev M et al. *Cold Spring Harb. Perspect. Biol.* (2013).

Figure 1.5: The mitochondrial microtubule motor complex

Illustrated here is a mitochondrion in blue (top) attached the mitochondrial tether (Miro, along with Milton) which connects the mitochondria to microtubule motor proteins (dyneins and kinesins) which are attached to the microtubules (blue, bottom).

Fig. 1.5

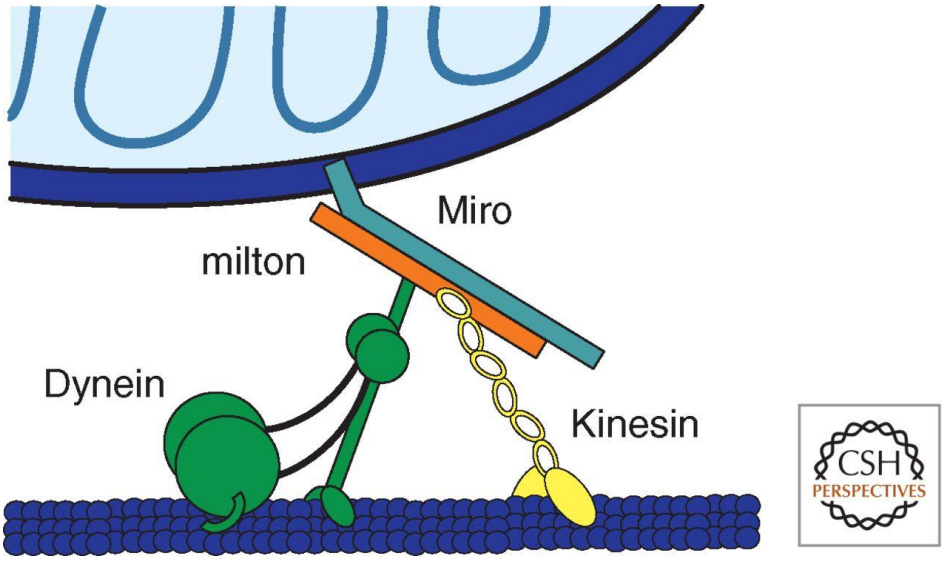


Image from Schwartz TL, *Cold Spring Harb. Perspect*. Biol. (2013)

Figure 1.6: Graphical representation of the three contexts of axon degeneration

Axotomy-induced Wallerian degeneration is induced by severing an axon to induce the selective degeneration of the axon distal to the cut site. Global NGF deprivation of young peripheral neurons induces axon apoptosis, where the entire neuron (both soma and axons) are degenerated. Pruning is the selective degeneration of only the axon exposed to the pruning stimulus, leaving the cell body intact. Pruning can be studied in compartmentalized (illustrated by the gray barrier) cultures where NGF can be selectively deprived from the axons, but not the cell bodies.

Fig. 1.6

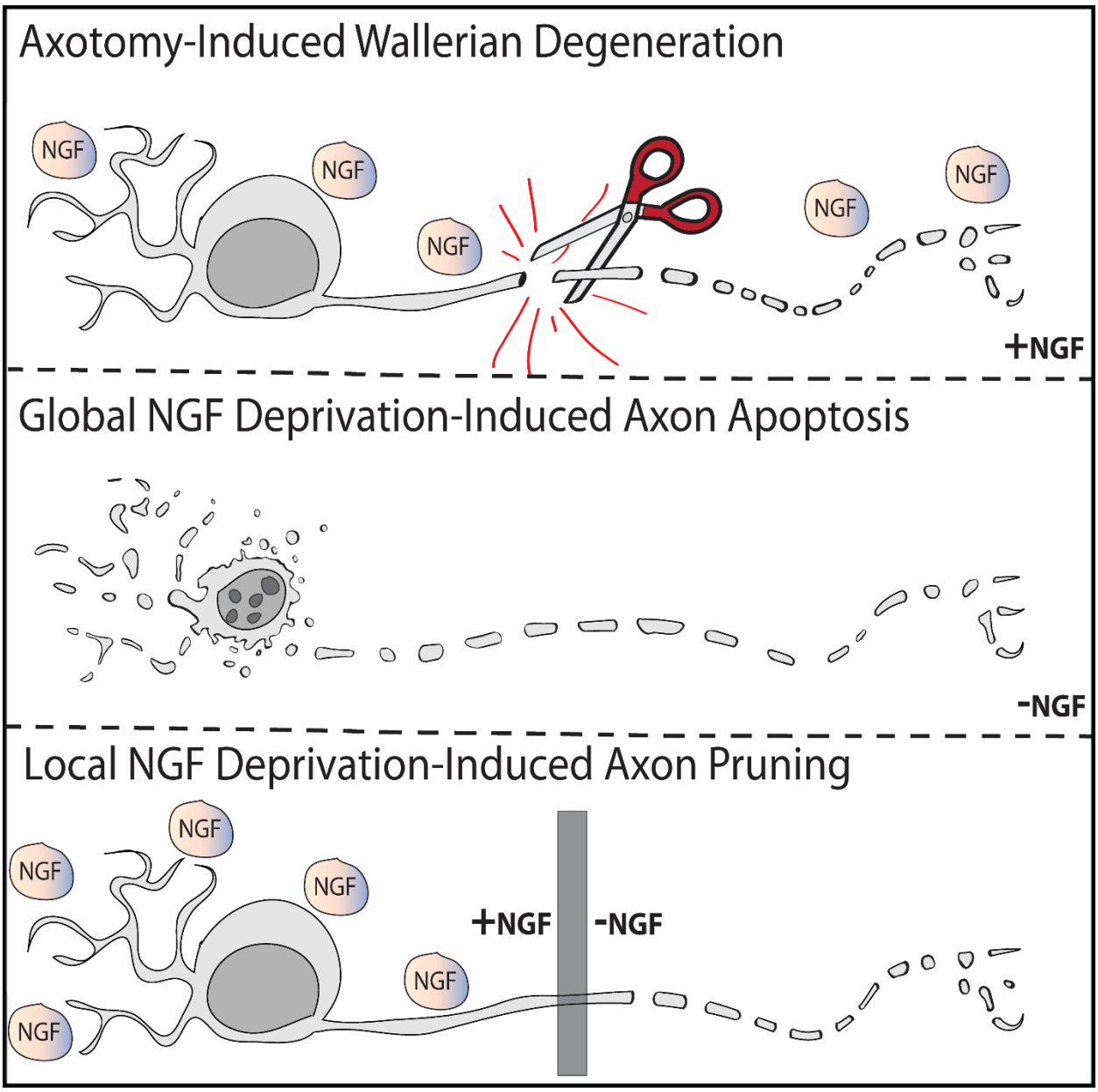

Axotomy-Induced Wallerian Degeneration
NGF
NGF
NGF
NGF
NGF
+NGF
Global NGF Deprivation-Induced Axon Apoptosis
-NGF
Local NGF Deprivation-Induced Axon Pruning
NGF
NGF
NGF
NGF
NGF
+NGF
-NGF

Table 1.1: Shared and distinct components of the axon degeneration pathways

Summary of the functional importance of select key proteins in mediating axon degeneration in the contexts of axotomy, apoptosis, and pruning.

The degree of protection reported is represented as: +++ excellent or complete protection, ++ significant protection, + some protection, - no protection, ? not yet known.

The relevant publications (not an exhaustive list) are also indicated.

Table 1:

	Axotomy	Apoptosis	Pruning
Wld^{s}	**+++** [50]	Axons **++** Soma **-** [48]	**?**
NMNAT overexpression	**+++** [51]	Axons **++** Soma **-** [49]	**?**
Sarm1 -/-	**+++** [13,14]	**+ / -** [13,14]	**?**
MLK-depletion (DLK, MLK, MEKK4)	**+++** [18,19,52]	**+++** [34,52,53]	**+++** [34]
Inhibition or loss of JNKs	**+++** [19,27]	**+++** [19,34]	**+++** [40]
Inhibition of Calpains	**+** [21,54]	**+** [21]	?
NGF/TrkA stimulation	**-** [27]	**+++** [27]	**+++** [27]
Bax -/-	**-** [55]	**+++** [56]	**+++** [38,42]
Apaf-1 -/-	**?**	**+++** [27,31]	**-** [42]
Caspase 9 -/-	**?**	**+++** [31]	**+++** [42]
Caspase 6 -/-	**?**	**-** [42]	**+++** [42,44]
Caspase 3 -/-	**-** [44]	**+++** [31]	**+++** [38,42]

CHAPTER 2: CISPLATIN INDUCED MTDNA DAMAGE DIRECTLY INDUCES AXON DEGENERATION IN PERIHPERAL NEURONS IN THE ABSENCE OF NUCLEAR DNA DAMAGE

2.1 Introduction

DNA damage-induced cellular pathways have been extensively studied not only because the maintenance of genomic integrity is fundamentally important in all organisms, but also because chemotherapeutic drugs that induce DNA damage are frontline treatments for cancer therapy (180). These DNA damaging events engage a number of downstream pathways depending on the degree of DNA damage. While low levels of DNA damage can induce cell cycle arrest, senescence or differentiation, excessive DNA damage can lead to cell death *via* apoptosis (181). This research has predominantly focused on the effects of DNA damage on the nucleus where the pathways engaged, including the nuclear DNA damage-induced apoptotic pathway, have been well characterized (182, 183). It is becoming increasingly evident that exposure of cells to DNA damaging drugs induces not just nuclear DNA damage but also mitochondrial DNA (mtDNA) damage. However, identifying the effects of mtDNA damage independent of nuclear DNA damage is challenging because of the difficulty in exposing only the mitochondria, and not nucleus, to DNA damaging drugs.

One current model of examining the role of mtDNA damage is the “mutator” mouse model where the mitochondrial DNA polymerase-gamma (POLG) has a mutation

which causes POLG to have a defective proof-reading domain (184, 185). These mutator mice slowly accumulate mtDNA damage from acquired errors incurred during mtDNA replication, incur increased oxidative stress, increased apoptosis, and exhibit an aging phenotype (185-187). Another model of mtDNA damage utilizes mitochondrially-expressed endonuclease restriction enzymes (188). The expression of these mtDNA targeted endonucleases induces double-stranded breaks, which also resulted in an aging phenotype. Both these mouse models underscore the importance of maintaining mtDNA fidelity and show that accumulated mtDNA mutations have the consequence of premature aging at the organismal level. However, these mouse models are not easily tractable for identifying the precise molecular pathways directly activated with mtDNA damaging drugs.

2.2 Results

Using microfluidic chambers to selectively induce mtDNA damage

To selectively induce mtDNA damage without any nuclear damage, WE have taken advantage of the structure of primary neurons where the cell body and axons are spatially distinct. While the cell body contains both nuclear and mitochondrial DNA, the axons do not have a nucleus and hence the only DNA in axons is mtDNA from the axonal mitochondria. Importantly, we used primary neurons in a microfluidic-based compartmentalized chambers, which provide the ability to isolate the neuronal cell bodies both fluidically and spatially from the axons (Fig. 2.1A). These microfluidic chamber devices have been rigorously demonstrated to maintain fluidic separation between the soma and axon compartments (169, 189). Importantly, exposure of only the axon chamber to DNA damaging agents is anticipated to induce selective damage to axonal mtDNA without affecting the nuclear DNA.

Exposure of cells to the DNA damaging drugs such as cisplatin is known to induce damage to both nuclear and mitochondrial DNA (136). Thus, we examined whether addition of cisplatin to only the axon chamber in our neuronal microfluidic model was capable of inducing mtDNA damage in the axonal mitochondria. Using an established PCR amplification assay for detecting mtDNA damage, we found that cisplatin exposure induced substantial damage to mtDNA in the axon chamber (Fig. 2.1B). Importantly, we used γH2AX staining, a marker of nuclear DNA damage (190), to examine whether the addition of cisplatin to only the axon chamber induced any nuclear DNA damage. γH2AX staining was virtually undetectable in the nucleus when cisplatin was added to only the axon chamber. In contrast, robust γH2AX staining was

observed in the nucleus as expected, when cisplatin was added to both the soma and axon compartments (Fig 2.1C, D). Together, these results show that addition of cisplatin to the axon chamber selectively induced mtDNA damage without inducing nuclear DNA damage.

Axonal mtDNA damage is sufficient to trigger axon degeneration

To determine if the induction of mtDNA damage in the absence of nuclear DNA damage was capable of inducing a degenerative or pathogenic state in cells, we added cisplatin to the axon compartment and monitored the axons over time. Strikingly, we found that exposure of cisplatin to only the axon compartment induced robust degeneration of the exposed axons. Consistent with the fact that addition of cisplatin in the axon compartment did not induce any DNA damage in the soma compartment, the axons and cell bodies in the soma compartment remained intact. We assessed this degeneration by immunofluorescent staining of the axons with tubulin and quantifying the degree of degeneration using an established assay for quantifying axon degeneration (191). In axons treated with cisplatin we observed greater than 90% axon degeneration while untreated axons remained healthy with minimal axon degeneration (Fig. 2.2A, B).

To confirm that our finding that mtDNA damage induces axon degeneration was broadly observed with multiple DNA damage-inducing drugs, we examined the consequences of exposing axons to camptothecin or d4T. While cisplatin induces DNA damage by the formation of a bulky covalent intrastrand crosslink, the Topoisomerase-I inhibitor camptothecin selectively binds the topoisomerase-I-DNA complex, resulting in

DNA strand breaks (192). Also, the nucleoside analog d4T, also known as stavudine, has been shown to be capable of damaging mtDNA by binding and inhibiting the function of the mitochondrial DNA polymerase-γ (193). Exposure of the axon compartment alone to camptothecin or d4T was also sufficient to induce axon degeneration as seen with cisplatin (Fig. 2.2C, D, E, F). Thus, exposure of axonal mitochondria to multiple modalities of DNA damage was sufficient to induce axon degeneration.

mtDNA damage-induced axon degeneration is not mediated by the pathways of apoptosis, axon pruning, or Wallerian degeneration

The observation that exposure of axons only to DNA damage triggers axon degeneration provides an opportunity to examine the degenerative pathway activated selectively with mtDNA damage. As discussed earlier, most previous studies have investigated the DNA damage-induced cellular pathways in a context where the entire cell (including both nucleus and mitochondria) is exposed to the DNA damaging drugs. In these contexts, DNA damage in neurons is known to induce cell degeneration *via* activation of the Bax- and Caspase-3 (Casp3) -dependent intrinsic apoptotic pathway (163, 194, 195). Briefly, DNA damage induces Bax activation that results in the mitochondrial release of cytochrome c leading to the activation of Casp3 and cellular degeneration. Neurons deficient in either Bax or Casp3 are protected from undergoing apoptosis when the entire cell is exposed to DNA damaging drugs (163, 194, 195). We examined whether this pathway was also important in mediating axon degeneration in response to selective mtDNA damage. Specifically, we isolated neurons from Bax- or

Casp3-deficient mice and evaluated their ability to undergo axon degeneration in response to axonal exposure to cisplatin. Surprisingly, neither the Bax- nor Casp3-deficient neurons exhibited any protection against cisplatin-induced axon degeneration (Fig. 2.3A, B, C, D). Thus, unlike the cellular degeneration induced by nuclear DNA damage, mtDNA damage induced axon degeneration does not appear to be mediated through the apoptotic pathway.

In neurons, two additional pathways of axon degeneration have also been identified. Axon degeneration in the context of axonal deprivation of nerve growth factor, activates the axon pruning pathway. While axon pruning, like apoptosis, also involves activation of caspases, axon pruning is reliant on the activity of an additional caspase, Caspase-6 (Casp6). To determine if any additional caspase-reliant pathways, such as axon pruning, are involved in mtDNA damage induced axon degeneration we used the pan-caspase inhibitor Q-VD-Oph hydrate (QVD). We found that QVD was unable to protect axons in response to axonal cisplatin exposure (Fig. 2.3E, F). These results indicate that neither the apoptotic nor the axon pruning pathways of programmed axon degeneration are required for mtDNA damage induced axon degeneration.

Axon degeneration in the context of axotomy is mediated via the Wallerian degeneration pathway. Wallerian degeneration is distinct from apoptosis or pruning and does not involve any caspase activation. Instead, the underlying mechanism of axon degeneration here is a consequence of catastrophic metabolic failure induced by the activity of Sarm1. As mtDNA damage would be expected to induce metabolic failure over time, we examined if axon degeneration observed in response to cisplatin occurred via Wallerian degeneration. To examine this, axons of neurons isolated from Sarm1-

deficient mice were exposed to cisplatin. However, Sarm1-deficient axons did not appear to be protected and degenerated in response to cisplatin (Fig. 2.3G, H). Thus, neither apoptosis not the two other well-recognized pathways of axon degeneration appear to be key mediators of mtDNA damage-induced axon degeneration.

Inhibition of ROS protects against mtDNA damage induced axon degeneration

As none of the identified pathways of axon degeneration appear to be required for the degeneration we observe in response to axon-only exposure to cisplatin, we decided to investigate alternative potential mechanisms of degeneration. Cisplatin is widely used as a chemotherapeutic drug in various cancer therapies. One of the well-known complications of cisplatin therapy is peripheral neuropathy where peripheral axons degenerate to cause significant neurological discomfort in patients. Interestingly, it has been reported that blocking the activity and generation of reactive oxygen species (ROS) using cell-permeable reduced glutathione (GSH) reduces the severity and incidence of peripheral neuropathies in patients treated with cisplatin (196-199). The exact mechanism by which cisplatin induces peripheral neuropathy and how GSH may protect is unknown. One possible mechanism of cisplatin-induced peripheral neuropathway is that axon degeneration is triggered in response to cisplatin-induced mtDNA damage in axons. To determine whether the mtDNA damage-induced axon degeneration we observe is potentially mediated via the aberrant production of ROS in the neuronal axons, we examined whether inhibiting the activity of ROS using the cell-permeable antioxidant GSH would confer protection. Interestingly, we found that the addition of GSH to only the axons in cisplatin-exposed axons provided significant

protection to axons from degeneration, reducing the measured degeneration from 89% axon degeneration in the cisplatin alone exposed axons to less than 6% axon degeneration in glutathione-saved and cisplatin treated axons (Fig. 2.4A, B).

2.3 Discussion

DNA damage-induced cellular pathway have been extensively studied not only because the maintenance of genomic integrity is fundamentally important in all organisms, but also because chemotherapeutic drugs that induce DNA damage are frontline treatments for cancer therapy (180). DNA damage induces repair pathways that can either result in cell survival, or if the extent of DNA damage is excessive, can trigger cell death (in certain contexts, DNA damage can also induce senescence or differentiation)(181). Justifiably, this research has predominantly focused on the effects of DNA damage on the nucleus where the nuclear DNA repair mechanisms or the nuclear DNA damage-induced apoptotic pathways have been well characterized (182, 183).

In contrast, our knowledge of the cellular pathways activated with mtDNAdamage is still limited. An important endogenous source of mtDNA damage are the reactive oxygen species (ROS), that are produced as a byproduct of mitochondrial oxidative phosphorylation (200). Spontaneous errors in the mtDNA replication machinery also contributes to mutations in mtDNA, which are known to accumulate with aging (85). Additionally, many exogenous agents such as ionizing radiation, environmental toxins, and chemotherapeutic drugs can also induce mtDNA damage. Most of our current knowledge about the cellular pathways activated with mtDNA damage has been focused on mtDNA repair mechanisms. For example, the primary mtDNA repair pathway is BER, although other repair mechanisms engaged include single strand and mismatch repair (45, 57, 85). Notably, mitochondria seem to lack the NER pathway, which is typically used to repair insults such as bulky adducts or cisplatin-induced

intrastrand crosslinks (45, 57, 85). As such, chemotherapy with cisplatin is known to induce substantial mtDNA damage, which is believed to not be repairable by the mitochondrial DNA repair machinery (201, 202).

Unlike irreparable nuclear DNA damage that actives a p53-dependent, apoptotic cell death program, less is known about the exact consequences of having irreparable mtDNA damage in cells. The main reason for this is that it is experimentally challenging to separate clearly the effects of nuclear *versus* mitochondrial DNA damage in cells. Other than the few notable exceptions highlighted below, most studies the have examined the molecular consequences of DNA damage in cells are in the context where the entire cell is exposed to the DNA damaging agent. In those situations, both nuclear and mitochondrial DNA are damaged, and the cellular outcome observed could be a combined effect of both nuclear and mitochondrial DNA damage pathways. Thus, to clearly define the cellular consequences mtDNA damage, there is a need to develop models where these chemotherapeutic drugs could selectively induce mtDNA damage without simultaneously also inducing nuclear DNA damage.

The best-studied model is the “mutator” mice, which carry a knock-in mutation in the exonuclease domain of the mitochondrial DNA polymerase, POLG, that renders the exonuclease domain defective. As this mutant POLG lacks its proof-reading activity, the mutator mice gradually accumulate mtDNA mutations and exhibit an aging phenotype (184, 185). An increase in oxidative stress and apoptosis is observed in these mice but the exact mechanism responsible for the aging phenotype is not known (185-187). Interestingly, the aging phenotype of these mice was recently shown to be rescued by physical exercise in a p53-dependent manner (203, 204). While the function

of p53 in the repair of nuclear DNA damage is well established (182), several reports show that p53 can also interact with mtDNA, enhance the fidelity of mtDNA replication, and maintain mtDNA genomic stability (205-208). Thus, mitochondrial homeostasis may not be efficiently restored by exercise in the absence of p53 in the mutator mice.

Another mouse model of mtDNA damage is one where mtDNA double-stranded breaks could be induced by the expression of a mitochondrially-targeted restriction endonuclease (Pst1) (209, 210). Even a transient induction of mtDNA double-stranded breaks is sufficient to induce an aging phenotype (211, 212). These mice exhibited an increase in the nuclear induction of p53 (and p21), but the aging phenotype could not be rescued with the deletion of p53 (or p21) (212).

Both these mouse models underscore the importance of maintaining mtDNA fidelity and show that accumulated mtDNA mutations have the consequence of premature aging at the organismal levels. However, these mouse models are not an easily tractable model to identify the precise molecular pathways directly activated with mtDNA damage. A few studies have induced mtDNA damage in cell lines. Depletion of the exonuclease EXOG, which resulted in persistent single stranded brakes in mtDNA (but not nuclear DNA), induced cell death with apoptotic characteristics, but the molecular components of the cell death pathway were not examined (188). Interestingly, the Kelley group has recently developed mitochondrially-targeted doxorubicin (213) and mitochondrially-targeted cisplatin (214) and find that addition of these to cancer cell lines induces cell death; the molecular mechanism of cell death was not examined in these studies.

Our model system of utilizing microfluidic chamber devices provides for the spatial and fluidic isolation of the neuronal cells bodies from the nucleus. This system is ideal for examining the effects of direct exposure of only the mitochondria, and not the nucleus, to DNA damaging drugs. Our data show that the axonal mitochondria exposed to cisplatin in our microfluidic devices incur mtDNA damage while the nucleus of the same cells remain undamaged. Our finding that cisplatin exposure to axons alone leads to significant axon degeneration while the untreated axons and nucleus remain intact is striking because it has not been shown before that DNA damage directed towards the mitochondria is capable of inducing degeneration without negative consequences to the nucleus. These data suggest that mtDNA damage is capable of directly leading to the downstream degeneration of the affected cellular material nearby the damaged mitochondria. It is notable that mitochondria completely lack the NER pathway for repairing more bulky adducts, among other types of mtDNA damage . As mitochondria lack key DNA repair pathways that are utilized in the nucleus for nuclear DNA maintenance and repair, it is likely that the mtDNA may be more susceptible to damage in response to certain DNA damage insults. Importantly, cisplatin intrastrand crosslinks that require the NER pathway, which is deficient in mitochondria, to be repaired (45, 81, 82). Our findings that both the topoisomerase I inhibitor camptothecin and the nucleoside analog d4T are capable of also inducing axon degeneration when exposed to axons alone suggests that the degeneration we observe is not specific to cisplatin induced mtDNA damage. Camptothecin and d4T induce DNA damage events through completely different mechanisms (likely through induction od double-stranded breaks), indicating that this is a conserved response to mtDNA damage.

There are a number of known pathways which can induce programmed cellular degeneration, but the three pathways that have been best studied in the context of axon degeneration are axon apoptosis, axon pruning, and Wallerian degeneration. Axon apoptosis and axon pruning share a number of the same mechanistic components including the requirement for Casp3, Bax, and caspase activity, although the two differ slightly (axon apoptosis requires Apaf-1 and not Caspase-6 while axon pruning requires Caspase-6 but not Apaf-1). As both axon apoptosis and axon pruning require Bax, which is known to permeabilize the mitochondrial membrane and induce energetic failure, it was attractive to consider that these pathways may play a role in mtDNA damage induced axon degenerations. Our results show that Casp3-decifienct or Bax-deficient neurons, or caspase inhibitor-treated neurons were incapable of significantly inhibiting axon degeneration in response to cisplatin-induced mtDNA damage. These data show that the axon apoptosis and axon pruning pathways of programmed axon degeneration do not seem to be the key essential degeneration pathways activated with axonal mtDNA damage. The third axon degeneration pathway that is well studied is the Wallerian degeneration pathway, which relies upon the key mediator Sarm1 to execute degeneration. One of the apparent roles of Sarm1 is to induce energetic failure by depletion of key metabolic intermediates and thus was potentially interesting in the context of mitochondrial damage. However, our findings with the Sarm1-deficient mice indicate that Sarm1 and the Wallerian degeneration pathways are also not essential mediators of cisplatin-induced axon degeneration.

Interestingly, despite none of the known axon degeneration pathways being involved we were surprised to find that the addition of the antioxidant glutathione

appeared to robustly protect the axons from undergoing axon degeneration. These data point to the involvement of ROS in the axon degeneration induced by cisplatin. The involvement of ROS in response to mtDNA damage is not surprising, as mtDNA damage in various other model systems has been shown to induce oxidative stress (105, 106). However, the ability of an antioxidant to robustly inhibit the degeneration, that was not prevented by the inhibition of key degenerative pathways, is interesting. In patients, it has been previously reported that the addition of glutathione was able to reduce some of the negative side-effects of cisplatin therapy. Our data suggest that glutathione addition appear to prevent the degeneration that is induced in response to mtDNA damage.

These findings are interesting because they could suggest new potential avenues of chemotherapeutic treatment therapies which could be designed to minimize CIPN. However, we also find it important to note that if one of the ways that DNA damaging therapies is working to kill cancer cells is *via* damaging mtDNA then the addition of glutathione might actually serve to protect cancer cells from the chemotherapeutic regime itself. Because little is understood about the exact contributions of mtDNA damage versus nuclear DNA damage in the outcomes of global cellular exposure we believe that additional study should be undertaken to further expand our knowledge on these roles. These findings indicate that the currently well-identified pathways of axon degeneration do not appear to be involved in the axon degeneration observed in response to cisplatin-mediated mtDNA damage. There are a number of potential explanations for this observations, ranging from the involvement of a less-appreciated pathway for axon degeneration or the implication of an unidentified

pathway. One interesting observation is that while the model system was in neurons and our findings were based on axon degeneration the consequences of mtDNA damage might be a more broadly applicable effect. We examined the known pathways of axon degeneration because those were the most rational pursuits within our system, but the outcomes of mtDNA damage may trigger and engages a more basal pathway that is maintained by all cell types. As mtDNA damage is a universal problem for all cells, it may stand to reason that the pathways engaged may too be universal. The cellular pathways engaged directly by mtDNA damage remain poorly understood and future research seeking to differentiate the contributions of mtDNA versus nuclear DNA damage to cellular outcomes will be valuable in expanding our knowledge on how mtDNA damage affects the fate of a cell.

2.4 Materials and Methods

Primary sympathetic neuronal cultures

Primary sympathetic neurons were cultured as previously described (164). Briefly, sympathetic neurons were dissected from the superior cervical ganglia (SCG) of postnatal day (P) 0-2 CD1 mice. Cells were plated directly into the microfluidic chamber device (described below) at a plating density of 10,000 cells per chamber and maintained for 5 days *in vitro* (DIV) in media containing NGF (AM50). Mice deficient for Bax, Casp3, Casp6, and Sarm1 are all from C57BL/6 backgrounds and wildtype littermates served as controls.

Fabrication and use of microfluidic devices

Microfluidic device master molds were generated using standard photolithographical procedures. Negative photoresist (MicroChem Corp, KMPR 1010) was utilized to pattern the chamber and microfluidic grooves. The microfluidic grooves were etched at 3 μm deep grooves with a groove width of 10 μm with each groove being spaced 40 μm apart into a standard silicon wafer (Silicon Question International). The raw silicon surrounding the groove patterns was etched approximately 3 μm into the silicon wafer using deep reactive ion etching (Alcatel AMS 100 Deep Reactive Ion Etcher) using a low-roughness Bosch process. The groove photoresist layer was then removed using 2800 W oxygen plasma. The axon and soma compartments (750 μm long, 100 μm deep, 100 μm wide for each side) were added on either side of the etched grooves by addition of a negative photoresist layer of SU-8 (MicroChem Corp) followed by standard photolithographic procedure recommended by the manufacturer. To

generate microfluidic devices, Sylgard 184 PDMS (Dow Corning) was prepared according to manufacturer protocol and poured into the mold form. The liquid mold was placed under vacuum to remove air bubbles from the PDMS for 30m then placed into a 65^0C oven to cure overnight. After curing, individual PDMS chambers were cut out and trimmed then soaked ddH2O overnight, sterilized in 70% EtOH, allowed to dry, then mounted onto cleaned glass coverslips coated overnight in 40 µg/ml poly-D-lysine (Sigma, P7886) and 0.1 µg/mL mouse laminin (Invitrogen, 23017-015).

Culture and treatment of primary neurons in microfluidic chambers

Neurons were plated in microfluidic chambers as described previously (169) and maintained in AM50 until 5 DIV (or 28 DIV for mature experiments). To treat cells with *Global* (both axon and soma chambers) drug treatment, both the axon and soma compartment are washed three times with AM50 media containing drug. To treat cells with *Local* (axon chamber only) drug treatment, only the axon compartment is washed with AM50 containing drug while the soma compartment is maintained in untreated AM50 media. A 30 µL volume differential was established between the soma and axon compartments and was reestablished every 12-20 hours to maintain fluidic isolation of the two compartments (169). For drug treatments, compounds were added to AM50 media at the following concentrations: 20 µM cisplatin (Sigma, P4394), 20 µM camptothecin (Tocris, #1100), 50µM d4T (Sigma, D1413), 25 µM QVD-OPh (SM Biochemicals, SMPH001). For experiments using NGF deprivation as a positive control for apoptosis, cultures were rinsed three times in media lacking serum (AM0) and then

maintained in NGF-deficient media containing a neutralizing antibody to NGF. All experiments were performed in triplicate.

Axon Degeneration Quantification

Degeneration of axons was quantified from immunofluorescent images stained for tubulin taken in the microfluidic devices. The images are analyzed in ImageJ using a published and accepted axon degeneration analysis based on axon segment continuity (191).

Immunofluorescence

Immunofluorescence staining was carried out as previously described (174). Briefly, the two compartments are stained by added solutions to the top chamber reservoirs and allowing solutions to flow through into the chamber areas. The following primary antibodies were used for staining: tubulin (Sigma, T9026), Tom20 (Santa Cruz, sc-11415), phospho-H2AX (Cell Signaling, 9718S). Nuclei were labeled with Hoechst 33258 (ThermoFisher, H3569).

Image acquisition and processing

Images were acquired on a DMIRE2 inverted fluorescent microscope (Leica) using an ORCA-ER B/W CCD camera (Hamamatsu) through Metamorph software (Molecular Devices, version 7.6).

mtDNA damage analysis

mtDNA was acquired by collecting axon-only mtDNA from the axon compartment of treated and untreated axons using a DNeasy Blood & Tissue kit (Qiagen, 69504). The mtDNA was then amplified using primers specific to short (117bp) and long (10kb) amplicons of murine mtDNA as previously described (215, 216). The 117bp short region used the following for amplification: forward primer: 5′-CCC AGC TAC TAC CAT CAT TCA AGT-3′, reverse primer: 5′-GAT GGT TTG GGA GAT TGG TTG ATG T-3′. The 10kb long region used the following for amplification: forward primer: 5′-GCC AGC CTG ACC CAT AGC CAT AAT AT-3′, reverse primer: 5′-GAG AGA TTT TAT GGG TGT AAT GCG G-3′. PCR was done using Platinum Blue TAQ (Invitrogen, 12580015) conditions for amplification were followed as previously described (216). The amplified mtDNA products were measured using a Picogreen DNA amplification assay as previously described using a Thermo Fluoroskan Ascent FL fluorescent plate reader (215). Resulting values of the short and long mtDNA amplification fragments were then ratiometrically compared as previously reported to quantify the amount of mtDNA damage in a sample (215, 216).

2.5 Figures and Legends

Figure 2.1: Cisplatin addition to only the axons in compartmentalized cultures induces axonal mtDNA damage without damaging nuclear DNA

(A) Illustration of the microfluidic chambers where neurons are placed in the soma compartment and allowed to extend their axons into the axon compartment through the microgrooves. **(B)** Neurons in microfluidic chambers were either left untreated (UNT) or treated with cisplatin to the axon chamber (Cis; axon only). Shown is quantification of mtDNA damage represented as % undamaged mtDNA in axonal mitochondria as compared to untreated controls (n=3). **(C)** Neurons in microfluidic chambers were left untreated (UNT) or treated with cisplatin to only the axon chamber (Axon only) or treated with cisplatin to both the compartments (Axon and Soma). Shown are images of the neuronal soma stained with antibodies to pH2AX (Top panels). Bottom panels show same images of nuclei stained with DAPI. While pH2AX foci in the nucleus are observed in globally treated neurons (cisplatin added to both axon and soma compartments), no pH2AX foci are visible upon cisplatin exposure to the axon-only compartment. **(D)** Quantification of pH2AX staining (n=3). All experiments were examined at 120 hours after treatment with 20 μM cisplatin.

Figure 2.1

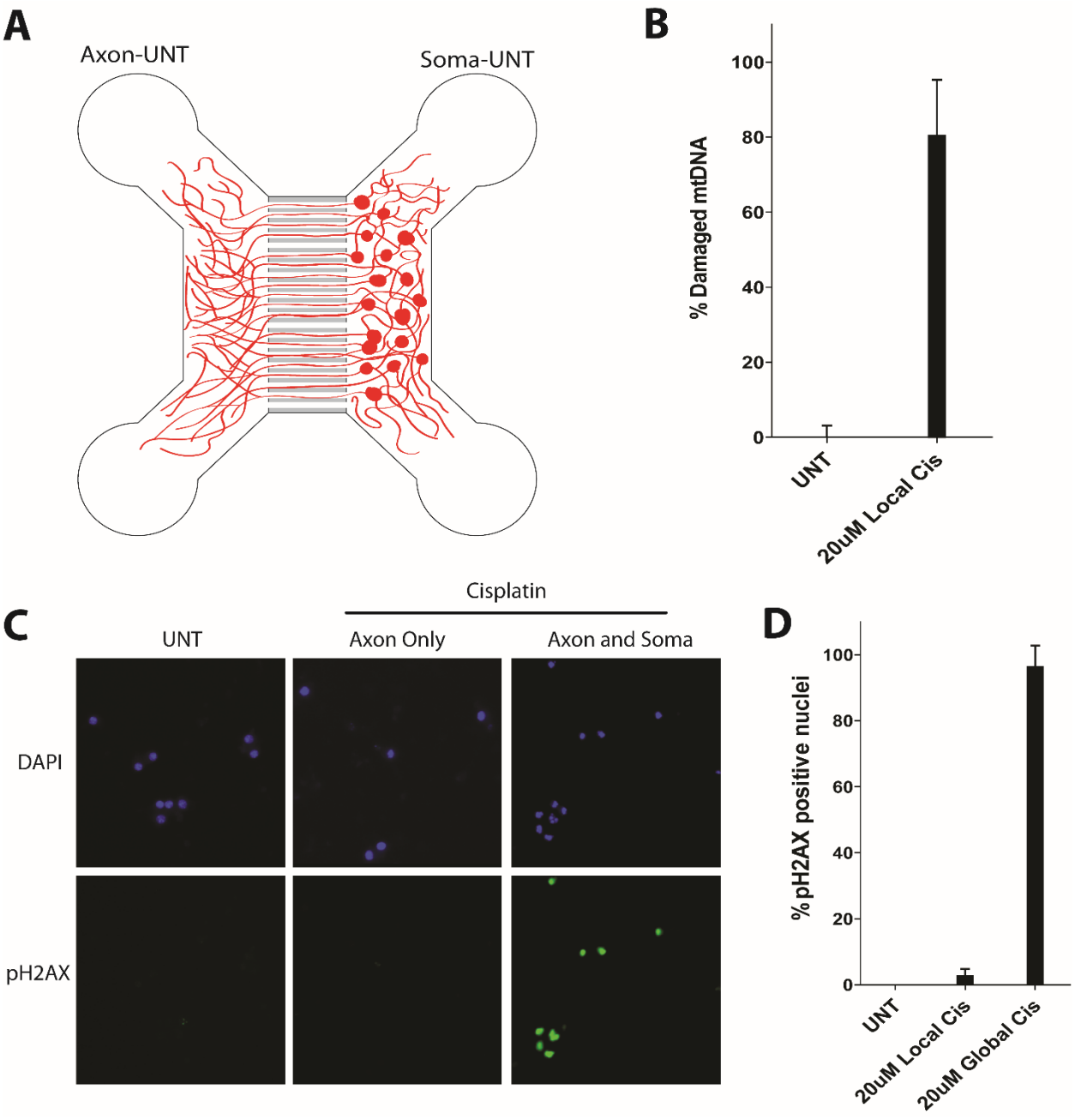

A
Axon-UNT
Soma-UNT
B
% Damaged mtDNA
100
80
60
40
20
0
UNT
20uM Local Cis
C
Cisplatin
UNT
Axon Only
Axon and Soma
DAPI
pH2AX
D
% pH2AX positive nuclei
100
80
60
40
20
0
UNT
20uM Local Cis
20uM Global Cis

Figure 2.2: mtDNA damage induced by cisplatin and other DNA damaging drugs in axons is sufficient to induce axon-specific degeneration.

(A) Neurons in microfluidic chambers were left untreated (UNT; Left panels) or treated with cisplatin to only the axon chamber (Local Cis, 20 μM; Right panels). Images of neurons stained with tubulin are shown at 120 hrs after treatment. **(B)** Quantification of cisplatin induced axon degeneration (n=3) from (A). **(C)** Neurons were treated in microfluidic chambers as in (A) with the nucleoside analog d4T (50 μM) for 120 hours and stained for tubulin. **(D)** Quantification of the d4T induced axon degeneration induced axon degeneration (n=3) from (C). **(E)** Neurons were treated in microfluidic chambers as in (A) with the topoisomerase inhibitor camptothecin (20 μM) for 120 hours and stained for tubulin. **(F)** Quantification of the camptothecin induced axon degeneration (n=3) from (E).

Figure 2.2

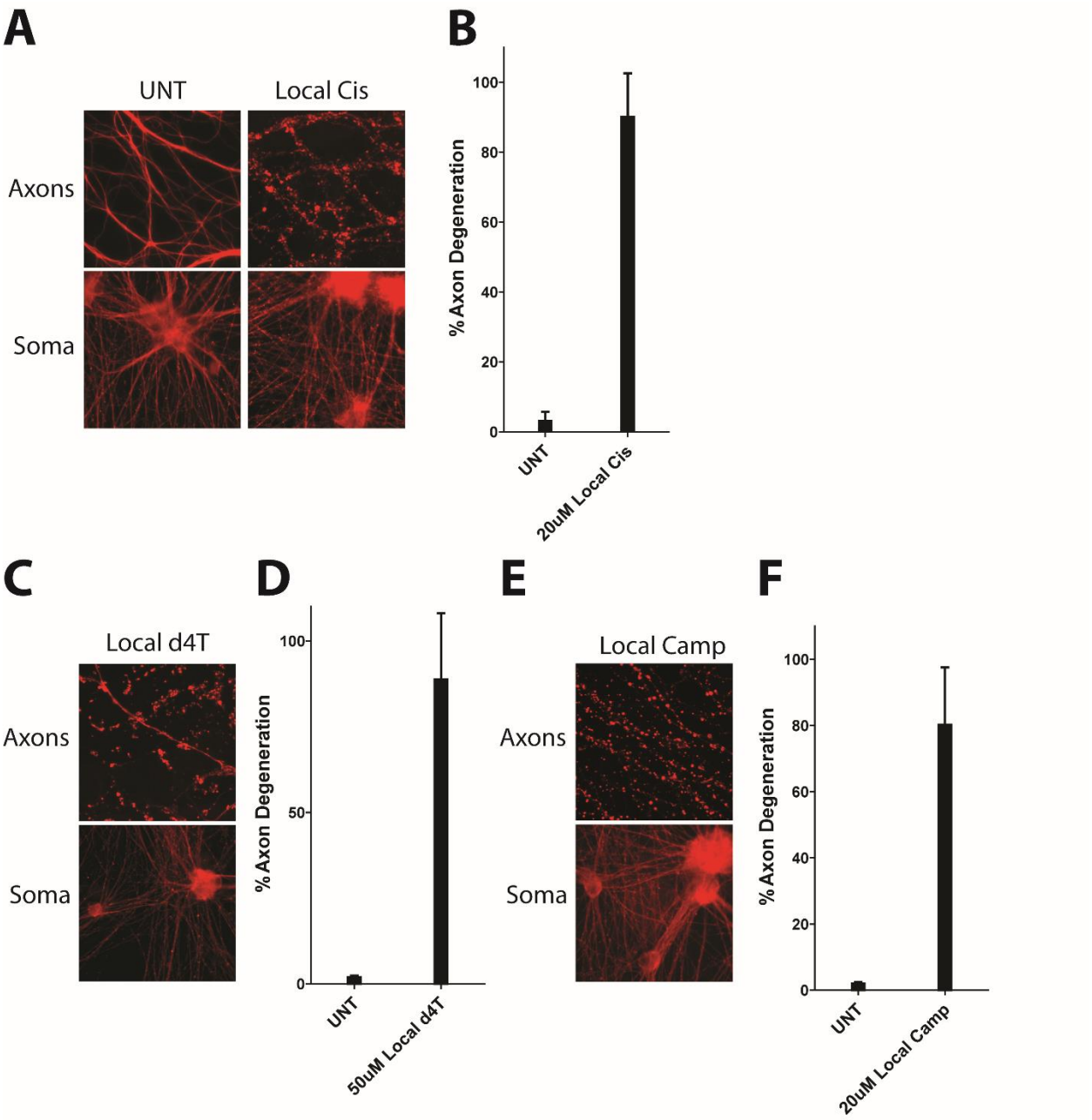

A
UNT
Local Cis
Axons
Soma
B
% Axon Degeneration
100
80
60
40
20
0
UNT
20uM Local Cis
C
Local d4T
Axons
Soma
D
% Axon Degeneration
100
50
0
UNT
50uM Local d4T
E
Local Camp
Axons
Soma
F
% Axon Degeneration
100
80
60
40
20
0
UNT
20uM Local Camp

Figure 2.3: Cisplatin-induced axon degeneration is mediated by the known pathways of axon degeneration.

(A) Neurons were isolated from Bax-deficient mice in microfluidic chambers and treated with cisplatin applied only to the axon chamber (Local Cis; 20 μM) for 120 hours and stained for tubulin. **(B)** Quantification of cisplatin induced axon degeneration (n=3) from (A). **(C)** Neurons were isolated from Casp3-deficient mice in microfluidic chambers and treated with cisplatin applied only to the axon chamber (Local Cis; 20 μM) for 120 hours and stained for tubulin. **(D)** Quantification of cisplatin induced axon degeneration (n=3) from (C). **(E)** Neurons cultured in microfluidic chambers were treated with cisplatin applied only to the axon chamber in the absence (Local Cis; 20 μM) or presence of QVD (Local Cis+QVD; 20 μM) for 120 hours and stained for tubulin. **(F)** Quantification of cisplatin induced axon degeneration (n=3) from (E). **(G)** Neurons were isolated from Sarm1-deficient mice in microfluidic chambers and treated with cisplatin applied only to the axon chamber (Local Cis; 20 μM) for 120 hours and stained for tubulin. **(H)** Quantification of cisplatin induced axon degeneration (n=3) from (G).

Figure 2.3

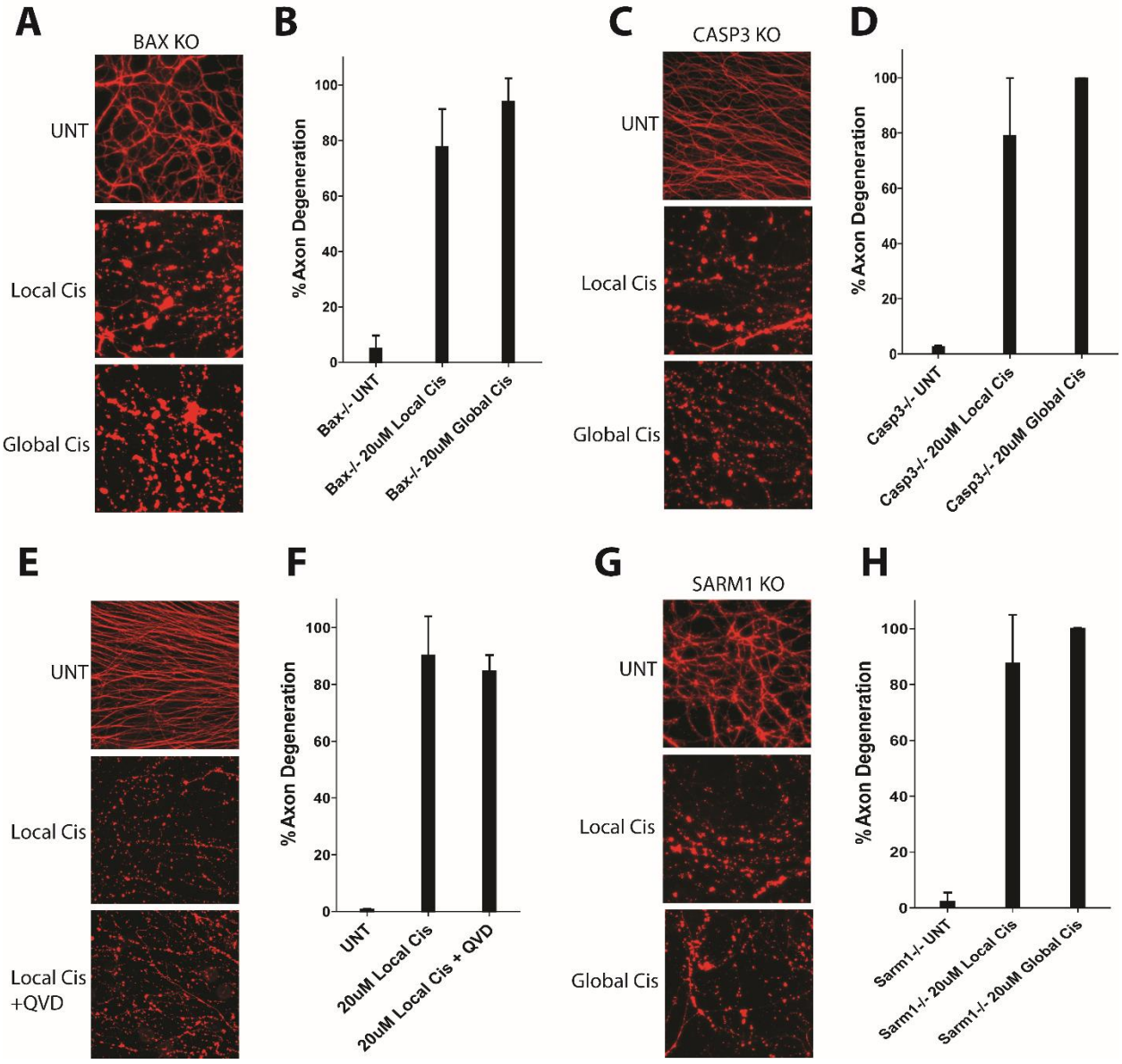
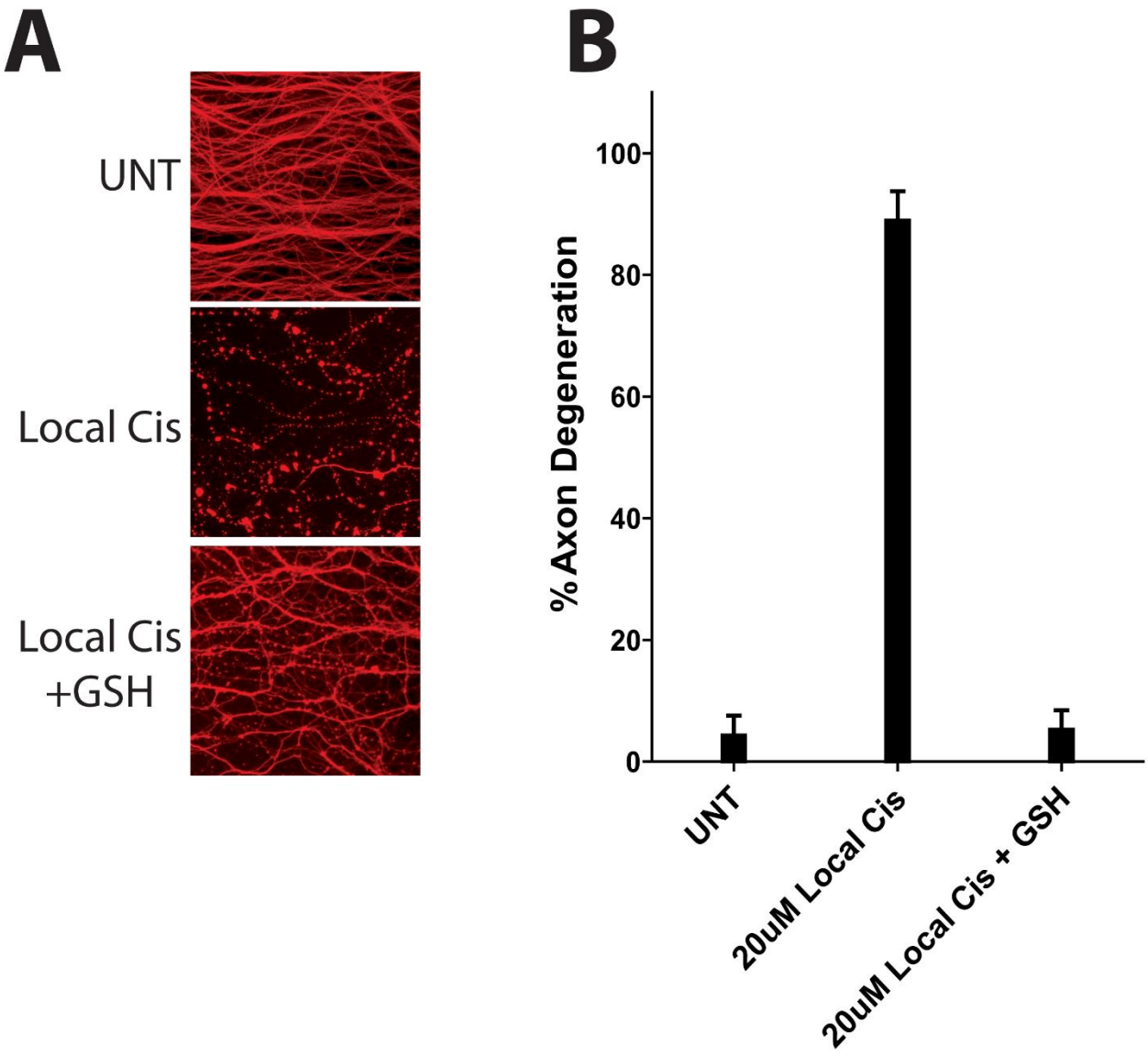

A
BAX KO
UNT
Local Cis
Global Cis
B
% Axon Degeneration
100
80
60
40
20
0
Bax-/- UNT
Bax-/- 20uM Local Cis
Bax-/- 20uM Global Cis
C
CASP3 KO
UNT
Local Cis
Global Cis
D
% Axon Degeneration
100
80
60
40
20
0
Casp3-/- UNT
Casp3-/- 20uM Local Cis
Casp3-/- 20uM Global Cis
E
UNT
Local Cis
Local Cis +QVD
F
% Axon Degeneration
100
80
60
40
20
0
UNT
20uM Local Cis
20uM Local Cis + QVD
G
SARM1 KO
UNT
Local Cis
Global Cis
H
% Axon Degeneration
100
80
60
40
20
0
Sarm1-/- UNT
Sarm1-/- 20uM Local Cis
Sarm1-/- 20uM Global Cis

Figure 2.4: Addition of the cell-permeable antioxidant glutathione inhibits cisplatin-induce axon degeneration.

(A) Neurons cultured in microfluidic chambers were treated with cisplatin applied only to the axon chamber in the absence (Local Cis; 20 μM) or presence of glutathione (Local Cis+GSH; 20 μM, 50 mM) for 120 hours and stained for tubulin. **(B)** Quantification of cisplatin induced axon degeneration (n=3) from (A).

Figure 2.4


A
UNT
Local Cis
Local Cis
+GSH
B
% Axon Degeneration
100
80
60
40
20
0
UNT
20uM Local Cis
20uM Local Cis + GSH

CHAPTER 3: EXAMINATION OF THE CONSEQUENCES OF DIRECTED MTDNA DAMAGE ON MITOCHONDRIA

3.1 Overview

In Chapter 2, we investigated the outcomes to the neuronal axon induced by the exposure of cisplatin as well as examined the potential pathways engaged during degeneration. Here, we examine the outcomes of mtDNA damage induced by cisplatin on mitochondria.

DNA damage in the nucleus, where the nuclear DNA repair mechanisms or the nuclear DNA damage-induced apoptotic pathways are involved, are well characterized (182, 183). In contrast, our knowledge of the cellular pathways activated with mtDNA damage is still limited. Specifically, because of the challenges in inducing mtDNA damage acutely and directly. The majority of cell culture models require the administration of damaging drugs to the entire cell, which prevents the examination of the consequence of mtDNA damage in the absence of nuclear DNA damage. As a result, the exact cellular consequences for direct and acute damage to the mtDNA are unclear.

MtDNA damage could lead to a variety of potential outcomes. For example, mitochondria with damaged mtDNA may become dysfunctional, depolarize, and become targeted for degradation *via* Parkin-mediated mitophagy (217, 218). This mitophagy is hallmarked by depolarization and aggregation of damaged mitochondria

that are to be eliminated. A number of potential consequences exist for mitochondria that become depolarized. If the damage is not severe, such compromised mitochondria may undergo repair in an attempt to regain membrane potential and functionality. Indeed, loss of membrane potential dislodges mitochondria from their microtubule motors while they decide whether to engage repair pathways or self-eliminate *via* mitophagy (120, 121). MtDNA damage also can lead to the production of ROS. An important form of mitochondrial ROS is superoxide, which is produced as a byproduct of mitochondrial oxidative phosphorylation, and is itself capable of damaging mtDNA (105, 106, 200). So while many potential outcomes exist for mtDNA damage, the exact consequences for directed mtDNA damage are not fully understood.

In this chapter, we document several aspects of mitochondrial biology and how mitochondria respond to directed mtDNA damage. These observations are ongoing and remain a work in progress.

3.2 Results and Discussion

Axonal cisplatin exposure leads to mitochondrial aggregation prior to degeneration

Having established that cisplatin is capable of inducing mtDNA damage and subsequent axon degeneration, we wanted to investigate the outcomes of mtDNA damage on mitochondria. As mtDNA damage occurs rapidly in response to cisplatin exposure (within 24 hours), we decided to examine mitochondria prior to degeneration (which occurs by 120 hours after damage). To determine whether cisplatin-induced mtDNA damage leads to mitochondrial deficits, we first examined if mitochondria underwent morphological changes in response to mtDNA damage. We added cisplatin to the axon compartment of neurons cultured in microfluidic chamber devices and examined their morphology at 72 hours post-exposure (prior to axon degeneration). Cisplatin-exposed axonal mitochondria appeared to show swelling and aggregation in response to treatment (Fig. 3.1A). When quantified, cisplatin exposure resulted in a significant reduction of numbers of individual mitochondria per unit area in the axon tracts (Fig. 3.1B). Accompanying the decreased mitochondrial abundance, the average size of each individual mitochondria was determined. We found that the average cisplatin-exposed mitochondria were more than twice the size of the average untreated mitochondria (Fig 3.1C). Reduced mitochondrial abundance and apparent increased mitochondrial aggregation (or fusion) suggest that fusion might be involved in the mitochondrial response to cisplatin-induced mtDNA damage (110). While mitochondrial fusion might be a viable mechanism to repair damage under physiological conditions, it relies on a large pool of healthy mitochondria to function as a repair pathway. The majority of mitochondria must be healthy and functional in order for the fusion of a defective mitochondria to healthy ones to be strongly justified. Under normal conditions,

where mitochondrial damage is likely restricted to a localized subset of the cellular mitochondria or to randomly distributed mitochondria, mitochondrial fusion is an adaptive repair approach. However, in response to catastrophic mitochondrial damage, such as cisplatin exposure, it could be predicted that the fusion of two failing mitochondria together would not provide sufficient regenerative effects to restore them to full functionality.

Cisplatin exposure causes mitochondria to lose the membrane potential

An alternate explanation to mitochondrial fusion for the observation that axonal mitochondria appear to be aggregated together in response to cisplatin induced mtDNA damage is that the mitochondria may be significantly compromised and have lost their membrane potential. A number of essential components of the mitochondrial ETC are encoded by the mtDNA. Thus, damage to mtDNA could prevent those essential ETC components from being produced and eventually lead to the breakdown of ETC functionality and subsequent loss of membrane potential. Upon loss of mitochondrial membrane potential, mitochondria would be then targeted for degradation by mitophagy (93). When mitochondria undergo mitophagy they are enveloped in a large double membrane structure and fused together with lysosomes. It is at this stage where they could appear to be large, highly fused structures when they are observed.

As mtDNA damage is known to induce deficits in the ability of mitochondria to effectively maintain their mitochondrial membrane potential, we examined if mitochondrial membrane potential was lost in response to cisplatin exposure (219). As

we have previously determined that mtDNA damage occurs within 24 hours of exposure and we know that mitochondria appear to aggregate by 72 hours of exposure, we examined mitochondrial membrane potential at the intermediate time point of 48 hours. Indeed, we found that cisplatin-exposed mitochondrial lost their mitochondrial membrane potential as measured by the mitochondrial membrane potential indicating dye, TMRE (Fig 3.2A). TMRE signal is lost in response to the direct exposure of mitochondria to cisplatin. These data indicate that mitochondrial membrane potential is lost within 48 hours of exposure to cisplatin, prior to the observation of mitochondrial aggregation and fusion.

Cisplatin-damaged mitochondria produce ROS in the form of superoxide

Damage to the mtDNA is known to lead to additional dysfunction of aerobic respiration, metabolic failure, and the production of ROS. Specifically, when mtDNA is damaged, ROS is produced in the form of the superoxide free radical from Complex I and Complex III of the mitochondrial ETC (100, 105, 106). As we found that mtDNA damage is induced by cisplatin exposure along with loss of mitochondrial membrane potential, we determined if cisplatin exposure to mitochondria led to generation of ROS. Indeed, we found that cisplatin treatment to neuronal axons led to marked production of ROS in the exposed mitochondria, as measured by the superoxide free radical (Fig. 3.3A). The formation of superoxide here suggests that there is likely significant damage to the mitochondrial ETC in response to cisplatin exposure. As superoxide is capable of damaging mtDNA by itself, these data suggest that the mtDNA damage induced by cisplatin can lead toward a feed-forward loop of further damage to the mtDNA by the consequent production of superoxide.

Cisplatin exposure leads to mitochondrial trafficking defects

It is known that damage to mitochondria and loss of mitochondrial membrane potential leads to the cessation of microtubule trafficking along microtubules (120, 121). As we have found that cisplatin exposure to mitochondria leads to significant mitochondrial damage and the loss of the mitochondrial membrane potential, we investigated if cisplatin led to mitochondrial trafficking defects. We used neurons isolated from the mitochondrially-targeted Dendra2 (mito-Dendra2) fluorescent reporter to conduct live-neuron imaging of mitochondrial movement in microfluidic chamber devices. We took a number of live-image video captures of mitochondrial movement after axonal exposure to cisplatin and found that almost all mitochondrial movement was inhibited. A representative image of one of these recordings is shown in Fig. 3.4A. Upon repeated analysis we were rarely able to observe any mitochondrial movement in cisplatin exposure axons, unlike the untreated axons which exhibited detectable mitochondrial movement patterns.

Mitochondrial depolarization leads to the phosphorylation of the mitochondrial tether protein, Miro, to microtubule motors that induces the destabilization and degradation of Miro (120, 121). Loss of the Miro tether to the microtubules motors prevents the trafficking of mitochondrial along the cytoskeleton. CIPN, including cisplatin-induced CIPN, has been known to manifest itself in a phenotype where the axons of peripheral neurons “die-back” towards the cell body (130).

As peripheral axons extend long beyond the cell body, they are increasingly exposed to the circulating fluids of the body, because axons have a high surface area to

cross-sectional ratio. In the case of a patient being treated with a chemotherapeutic agent, those fluids will contain that agent. Mitochondria that are physically present at the distal tip of a peripheral axon have been trafficked all the way from the cell body and out to the axon tip. Those mitochondria along their journey to the axon tip, or back from the axon tip, may be exposed to significantly higher doses of a chemotherapeutic drug, such as cisplatin, than mitochondria more proximal to the cell body. If those mitochondria were exposed to threshold doses of DNA-damaging drug on their anterograde journey then they may accumulate sufficient mtDNA damage to lead to the depolarization of their mitochondrial membrane and thus termination of movement along the axonal microtubules. This means that the newly generated healthy mitochondria produced in the neuronal cell body could become damaged as they traffic out towards a damaged axon tip. This progressive damage of the most distal mitochondria would manifest in a gradient of damage, where the most distal mitochondria to the cell body are the most damaged while the most proximal are the least affected. As CIPN induced by cisplatin manifests in this way, it is possible that this may be a potential mechanism behind the “dying-back” disease manifestation. The data we report here do not directly prove this hypothesis, but are consistent with it.

Induction of mtDNA damage leads to increased mtDNA replication

In this chapter we have examined the effects of cisplatin-induced mtDNA damage on mitochondria by primarily investigating the degenerative pathways involved. However, mitochondria likely exist in a more biologically dynamic state where they balance repair and survival against death and degeneration. When an entire cell is

exposed to DNA damage, the cell makes a number of decisions about whether the damage is tolerable, whether the damage is repairable, or if it is so excessive that it should eliminate itself through apoptosis. We wanted to determine if mitochondria attempted to alleviate damage to mtDNA by replicating themselves and expanding in response to mtDNA damage. Here, we utilized a plasmid expressing a component of the mitochondrial POLG polymerase which, when expressed, is selectively localized to only the mitochondria undergoing actively mtDNA replication (220). As a proof-of-concept, we looked in HeLa cells to find that cisplatin exposure appears to induce mtDNA replication as there is an increased number of mitochondria which costains with the POLG1 fluorescent reporter (Fig 3.5A). These findings are very preliminary and in future experiments our plan is be to use this plasmid construct in primary neurons in the microfluidic culture system. There, we would be to examine whether damage to axonal mitochondria induced increased mitochondrial proliferation in the exposed axons, the unexposed cell body, or both.

3.3 Materials and Methods

Primary sympathetic neuronal cultures

Primary sympathetic neurons were cultured as previously described (164). Briefly, sympathetic neurons were dissected from the superior cervical ganglia (SCG) of postnatal day (P) 0-2 CD1 mice. Cells were plated directly into the microfluidic chamber device (described below) at a plating density of 10,000 cells per chamber and maintained for 5 days *in vitro* (DIV) in media containing NGF (AM50). The mito-Dendra2 fluorescent reporter mice were acquired from Jackson Labs.

Fabrication and use of microfluidic devices

Microfluidic device master molds were generated using standard photolithographical procedures. Negative photoresist (MicroChem Corp, KMPR 1010) was utilized to pattern the chamber and microfluidic grooves. The microfluidic grooves were etched at 3 μm deep grooves with a groove width of 10 μm with each groove being spaced 40 μm apart into a standard silicon wafer (Silicon Question International). The raw silicon surrounding the groove patterns was etched approximately 3 μm into the silicon wafer using deep reactive ion etching (Alcatel AMS 100 Deep Reactive Ion Etcher) using a low-roughness Bosch process. The groove photoresist layer was then removed using 2800 W oxygen plasma. The axon and soma compartments (750 μm long, 100 μm deep, 100 μm wide for each side) were added on either side of the etched grooves by addition of a negative photoresist layer of SU-8 (MicroChem Corp) followed by standard photolithographic procedure recommended by the manufacturer. To generate microfluidic devices, Sylgard 184 PDMS (Dow Corning) was prepared

according to manufacturer protocol and poured into the mold form. The liquid mold was placed under vacuum to remove air bubbles from the PDMS for 30m then placed into a 65^0C oven to cure overnight. After curing, individual PDMS chambers were cut out and trimmed then soaked ddH2O overnight, sterilized in 70% EtOH, allowed to dry, then mounted onto cleaned glass coverslips coated overnight in 40 µg/ml poly-D-lysine (Sigma, P7886) and 0.1 µg/mL mouse laminin (Invitrogen, 23017-015).

Culture and treatment of primary neurons in microfluidic chambers

Neurons were plated in microfluidic chambers as described previously (169) and maintained in AM50 until 5 DIV (or 28 DIV for mature experiments). To treat cells with Global (both axon and soma chambers) drug treatment, both the axon and soma compartment are washed three times with AM50 media containing drug. To treat cells with Local (axon chamber only) drug treatment, only the axon compartment is washed with AM50 containing drug while the soma compartment is maintained in untreated AM50 media. A 30 µL volume differential was established between the soma and axon compartments and was reestablished every 12-20 hours to maintain fluidic isolation of the two compartments (169). For drug treatments, compounds were added to AM50 media at the following concentrations: 20 µM cisplatin (Sigma, P4394).

Immunofluorescence

Immunofluorescence staining was carried out as previously described (174). Briefly, the two compartments are stained by added solutions to the top chamber reservoirs and allowing solutions to flow through into the chamber areas. The following

primary antibodies were used for staining: tubulin (Sigma, T9026) Tom20 (Santa Cruz, sc-11415) Nuclei were labeled with Hoechst 33258 (ThermoFisher, H3569). Quantification of mitochondrial size and number was conducted using the ImageJ (NIH) particle count program.

TMRE assay for mitochondrial membrane potential

TMRE (Abcam) working stock solution was made up fresh from frozen stocks stocks immediately prior to use. A working concentration of 50nM TMRE was made up in normal AM50 neuronal cell media. Neuronal cell media was removed and replaced with the 50nM TMRE solution in AM50 and cells were replaced into the humidified 37^{o}C incubator for 15 minutes. After 15 minutes, cells were removed from the incubator and had the TMRE solution gently removed and replaced with PBS as per manufacturer's instructions. Cells were then imaged by fluorescent microscope.

MitoSOX Red superoxide detection assay

MitoSOX Red (Molecular Probes) was thawed and made up just prior to use using DMSO to make a 5mM stock which was then diluted to a working stock of 5 μM made up in neuronal AM50 media. Neuronal culture media was removed and replaced with 5 μM MitoSOX Red working stock and the cells were incubated for 10 minutes in a humidified 37^{o}C incubator. After 10 minutes, the MitoSOX Red solution was removed, and the neurons were washed three times with prewarmed PBS and then immediately imaged by fluorescent microscope.

mtDNA replication assay

The POLG fluorescent images were acquired by expression of the POLG1-GFP (also known as p55-GFP) plasmid, which was received as a kind gift from Dr. William C. Copeland (NIEHS) (220). Experiments were done using HeLa cell lines (ATCC) which were cultured in DME media (Gibco) supplemented with 10% fetal bovine serum (Sigma) and 2mM glutamine (Gibco). Adherent cells were passaged 1:10 using 0.25% trypsin (Gibco) when they were 70% confluent and treated with cisplatin (Sigma, P4394) at 70% confluence for 8 hours. All cells were maintained at 37^{o}C in humidified incubators containing 95% air and 5% CO_2.

Image acquisition and processing

Images were acquired on a DMIRE2 inverted fluorescent microscope (Leica) using an ORCA-ER B/W CCD camera (Hamamatsu) through Metamorph software (Molecular Devices, version 7.6). Live cell imaging videos were acquired through this setup using the Metamorph software.

3.4 Figures and Legends

Figure 3.1: Axonal exposure to cisplatin induces mitochondrial aggregation in neuronal axons.

(A) Cisplatin (20 μM) is added to the axons of neurons cultured in compartmentalized culture, where mitochondrial aggregation is observed. Arrows (red) indicate health mitochondria on the left untreated condition (NT), while arrows on the right point to aggregated mitochondria in the cisplatin exposed axons (Cisplatin). Neurons were exposed to cisplatin for 72 hours, then stained for the mitochondrial marker Tom20. (B) Quantification of the total number of mitochondria per unit area in healthy axons (Untreated) are compared to the number of mitochondria in locally treated axons (Local cisplatin) of the experiments done in (A) (n=2). (C) Quantification of the average size of mitochondria shown in (A) (n=2).

Figure 3.1

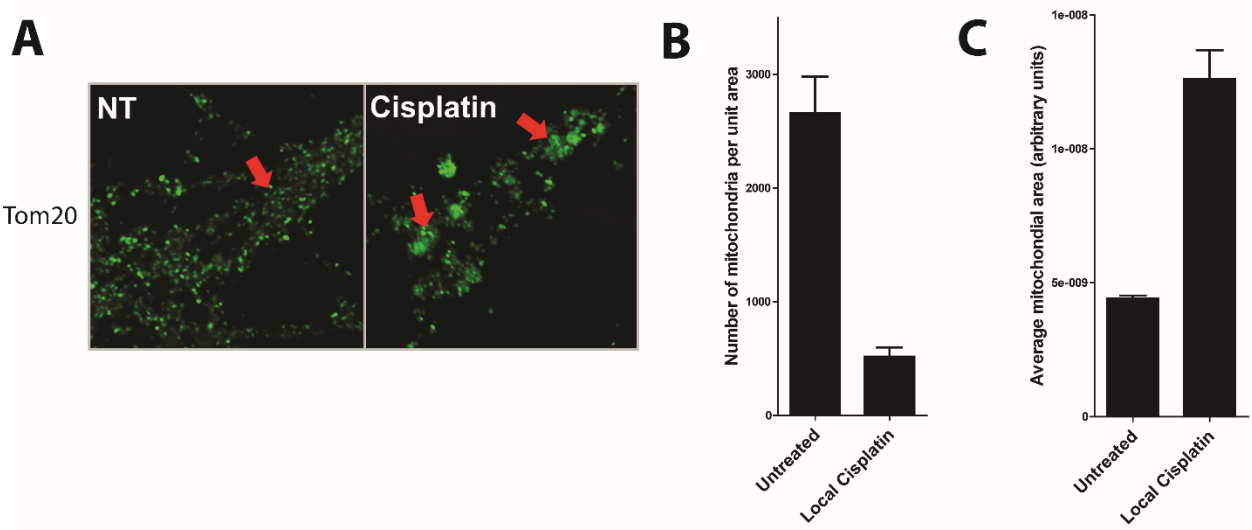

A
NT
Cisplatin
Tom20
B
Number of mitochondria per unit area
3000
2000
1000
0
Untreated
Local Cisplatin
C
Average mitochondial area (arbitrary units)
1e-008
1e-008
5e-009
0
Untreated
Local Cisplatin

Figure 3.2: Cisplatin induces depolarization of axonal mitochondria

(A) Axons of neurons cultured in microfluidic chamber devices were exposed to vehicle (UNT) or to cisplatin (Cis, 20 μM) for 48 hours. The axons were then probed with the mitochondrial membrane potential assay of TMRE retention. Mitochondria which maintain their membrane potential retain the red TMRE dye, depolarized mitochondria are unable to retain TMRE and appear black. Phase contrast images of axons are provided to indicate the location of axons.

Figure 3.2

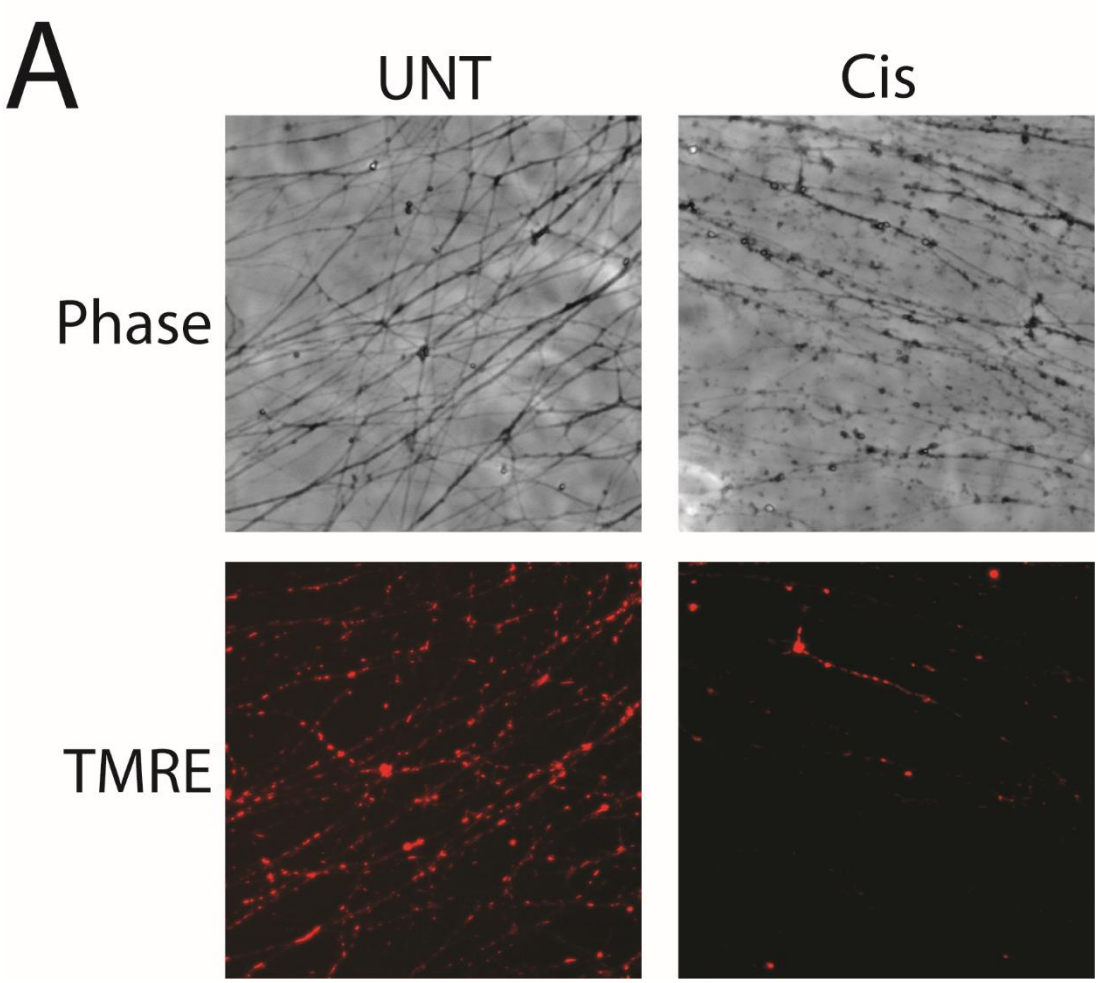


Figure 3.3: Cisplatin exposure induces the production of ROS

(A) Axons of neurons cultured in microfluidic chamber devices were exposed to vehicle (UNT) or to cisplatin (Cis, 20 μM) for 48 hours. The axons were then probed for the production of superoxide using MitoSOX Red. Red staining indicates the production of superoxide reactive oxygen species. Phase contrast images of axons are provided to indicate the location of axons. (n=2)

Figure 3.3

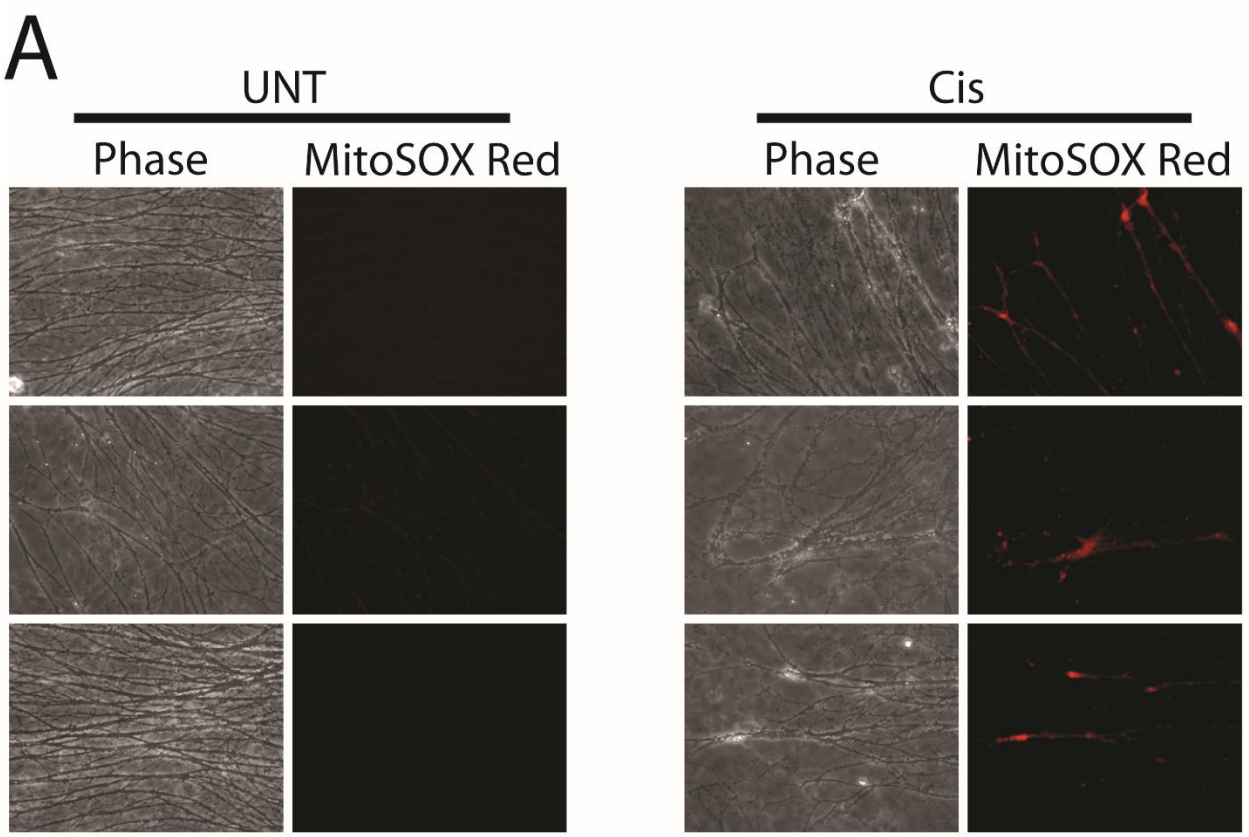


Figure 3.4: Cisplatin exposure disruptions mitochondrial trafficking

(A) Axons of neurons isolated from mito-Dendra2 mice were cultured in microfluidic chamber devices and exposed to vehicle (UNT) or to cisplatin (Cis, 20 μM) for 96 hours. Live cell video recordings were taking over 10 minute time periods of one frame acquired per 5 seconds. Here, a mitochondria indicated by a red arrow in the untreated condition is show trafficking down an axon. No mitochondria in the cisplatin-exposed axons were measured to be trafficked, and this lack of motion is noted by the marking of a representative mitochondria which fails to traffic (n=2).

Figure 3.4

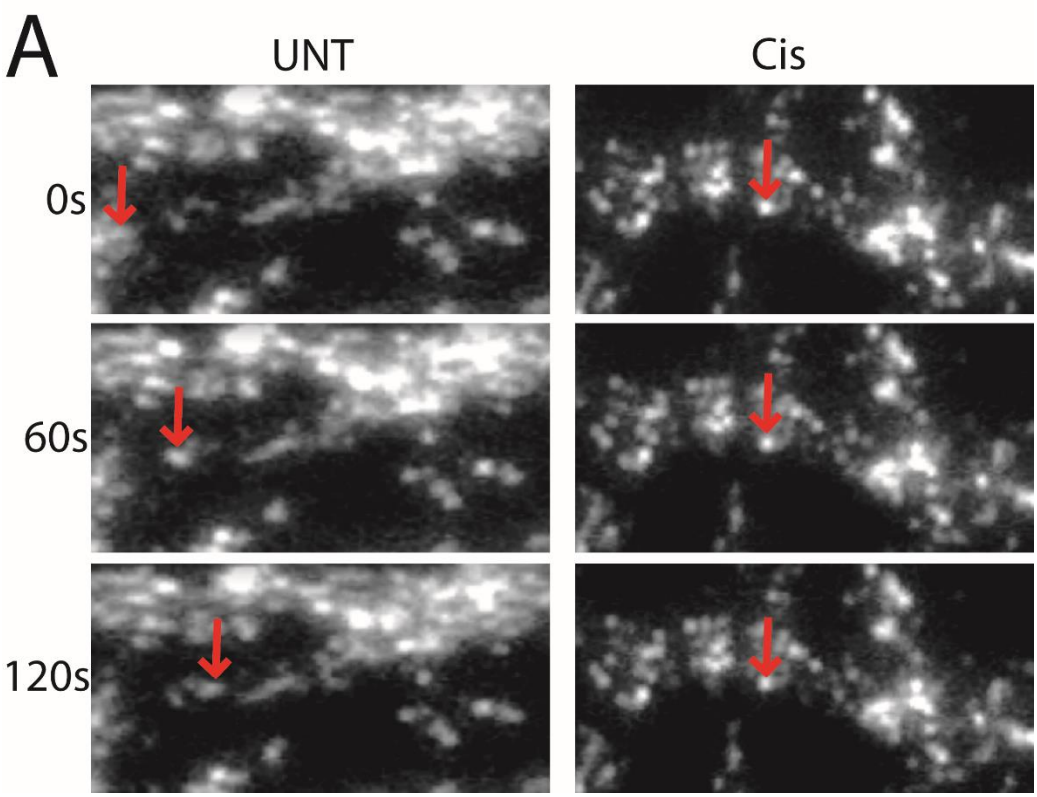
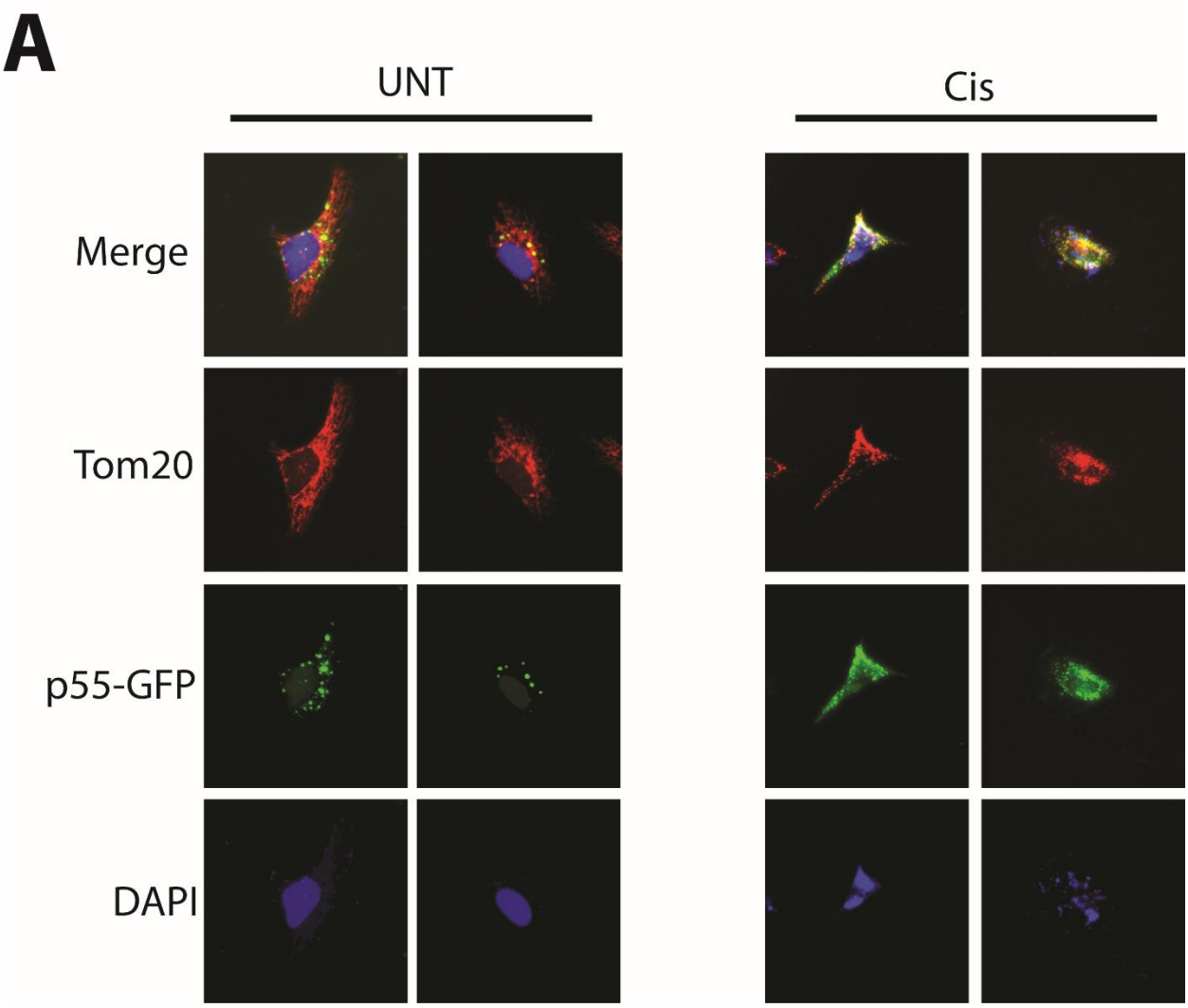


Figure 3.5: Cisplatin exposure induces the attempted replication of mtDNA in HeLa cells.

(A) HeLa cells were transfect with the POLG1/p55-GFP plasmid of the POLG holoenzyme and were exposed to vehicle (UNT) or cisplatin (Cis; 10 μM) for 8 hours. Cells were then fixed and stained for Tom20 and DAPI, representative images are shown here (n=1).

Figure 3.5



CHAPTER 4: DISCUSSION

4.1 Summary of findings

Cisplatin-induced mtDNA damage, in the absence of nuclear DNA damage, induces cellular degeneration

Almost all cells rely upon healthy and functioning mitochondria for the maintenance of cellular survival and viability. These mitochondria serve a number of key functions for cells, ranging from maintaining calcium ion buffering, fatty acid oxidation, Krebs cycle, and essential metabolic pathways (221). Due to their critical importance, cells have developed a number of processes by which to check for mitochondrial health and ensure repair or elimination of defective mitochondria. Indeed, defects in these programmed pathways of mitochondrial maintenance are implicated in a number of diseases, such as in the case of mitophagy defects and Parkinson's disease (99). One aspect of mitochondrial biology that remains unclear is what are the exact cellular and mitochondrial consequences of mtDNA damage. The cellular consequences of DNA damage, to both to the nuclear and the mitochondrial DNA, has been investigated. However, these studies rarely are able to selectively examine the pathways engaged directly in response to mtDNA damage, and in the absence of nuclear DNA damage.

In order to investigate the consequences of mtDNA damage alone, we utilized microfluidic chambers that allow for the spatial and fluidic isolation of the neuronal axons from cell bodies. This system allowed us to expose only the axon−and not the cell body−to the DNA damaging agents and examine the outcome of inducing selective damage to mtDNA independent of any damage to the nuclear DNA. All work unless indicated otherwise was done in the paradigm of drug addition to only the axons and not the cell bodies of neurons cultured in compartmentalized cultures. The main findings of this work are summarized below:

1. Exposure of cisplatin to axonal mitochondria induced mtDNA damage.
2. Nuclear DNA was not damaged when cisplatin was added to the axons alone (as indicated by pH2AX staining).
3. Cisplatin exposure to axons was capable of inducing axonal degeneration, without loss of the cell body and proximal, unexposed, axons.
4. To confirm that the mtDNA-induced degeneration was not specific to cisplatin, we confirmed that addition of the DNA damaging nucleoside analog d4T was also capable of inducing axon degeneration without loss of the cell body.
5. Additionally, we confirmed the degeneration phenotype using a third DNA damaging agent, the topoisomerase I inhibitor camptothecin. Exposure of camptothecin was also able to induce axon degeneration without loss of the cell body.
6. The axon degeneration induced by cisplatin is not mediated by the apoptotic pathway.

7. The axon degeneration induced by cisplatin is not mediated by the axon pruning pathway.
8. The axon degeneration induced by cisplatin is not mediated by the Wallerian degeneration pathway.
9. The degeneration however is able to be inhibited by the addition of exogenous antioxidants in the form of cell-permeable glutathione.
10. Addition of cisplatin in axons appeared to induce mitochondrial aggregation, with reduced total numbers of mitochondria in axons while the average size of each individual mitochondria increased.
11. The mitochondrial membrane potential appeared to be lost in response to mitochondrial cisplatin exposure, prior to axon degeneration.
12. Mitochondria appear to produce ROS, in the form of the superoxide free radical, in response to mitochondrial cisplatin exposure.
13. Mitochondrial trafficking appeared to be inhibited by cisplatin exposure, as determined by analysis of live cell imaging of axonal mitochondrial.
14. Preliminary data in HeLa cells using a POLG1-GFP reporter indicated that cisplatin exposure may induce the replication of mtDNA.

4.2 Clinical relevance

As damage to mtDNA is something that can occur in any cell type throughout its existence, these findings have relevance to a number of potential clinical conditions and human health. Here, we will discuss how these findings connect with human diseases and the potential meaning of these findings.

Chemotherapy-induced peripheral neuropathies

As previously discussed, one serious consequence of a number of chemotherapeutic regimes is CIPN. CIPN is a progressive and persistent neurodegenerative disorder induced by chemotherapeutic treatment. Up to 30-40% of patients undergoing chemotherapy are likely to encounter some form of CIPN during their treatment (125). As CIPN can induce a variety of symptoms in the outer extremities (e.g. hands, feet) ranging from numbness, tingling, thermosensitivity, to extreme pain or loss of sensations (222). These symptoms sometimes can cease after the end of treatment, but can often lead to enduring and irreversible neurological damage.

The exact cause of CIPN is not exactly clear, but it appears that different drug classes may have different disease etiologies. For example, the microtubule-disrupting chemotherapeutic taxanes (and vincristine) have been predicted to destabilize microtubules in neuronal axons (125). Neurons are dependent upon functional microtubule networks for their function, survival, and maintenance. Thus, it could be expected that disruption of these key networks potentially would lead to neurological deficits. Other chemotherapeutic drugs such as cisplatin, carboplatin, and oxaliplatin

are all known to induce CIPN but have a less clear mechanism for inducing their neuropathic effects. They have been shown to be able to induce some mtDNA damage, but the exact consequences of this damage has been unclear (222). Some studies have shown that cisplatin, among others, was capable of increasing ROS production, damaging mtDNA, and disrupting mitochondrial function (222, 223). It is important to note however that in these studies, cisplatin was exposed to the entire animal or to the entire cell. As global exposure to DNA damage could induce a number of consequences, this experimental design makes it challenging to determine if these outcomes are induced by a number of potential causes including neuronal nuclear DNA damage, damage to supportive Schwann cells, induced by demyelination, and damage to mtDNA.

In our studies, we found that unmodified cisplatin was able to directly induce mtDNA damage in primary peripheral neuronal axons, without any detectable damage to the nuclear DNA. Additionally, as exposure of axonal alone to cisplatin was capable of inducing axon degeneration in this context these data suggest that the dying-back degeneration observed in CIPN might be a direct consequence of mtDNA damage. Our findings that other DNA damage drugs were able to recapitulate our findings with cisplatin additionally supports the concept that sufficient mtDNA damage can lead to the degeneration of axons in CIPN. However, we were not able to identify any of the known pathways of axon degeneration (apoptosis, axon pruning, and Wallerian degeneration) as being involved in this degeneration. While inhibition of ROS did protect axons from undergoing cisplatin-induced axon degeneration it is not clear how this effect is mediated. One potential option could be that mtDNA damage induces ROS production

which is the driver of degeneration and that inhibition of ROS is sufficient to protect against degeneration. An alternate hypothesis is that the antioxidant addition actually prevents cisplatin from being able to induce mtDNA damage in the first place which would temper the initial damage as well as the ROS production. Indeed, this remains an important clinical point as GSH has been given to patients undergoing cisplatin therapy (198, 224). These studies have found that the addition of GSH indeed reduce the incidence of CIPN in patients. However, if the possibility exists that GSH prevents cisplatin from being able to properly function in its role as a chemotherapeutic agent for cancer therapy then these therapies should undergo additional review to ensure they do not change patient outcomes to the therapy. There may be other potential strategies however that could be designed to selectively mitigate damage to neuronal mitochondrial or mitochondria in general, which could provide a more targeted mechanism to reduce CIPN incidence without effecting patient cancer therapy outcomes.

Damage to mtDNA as a potential mechanism for the effects of chemotherapeutic drugs

While we have primarily focused here on the effects of selective mtDNA damage, it is worth considering these data in a broader context. We found that mtDNA damage was able to directly induce cellular degeneration of the neuronal axon when exposed to cisplatin. This suggests that damaged mtDNA could play a role in the degeneration of all cells exposed to DNA damage. Normal healthy cells are generally thought to engage p53-mediated apoptosis in response to significant DNA damaging insults. However, cells that have defective DNA damage response pathways, such as cancer cells, may

not engage in those apoptotic pathways to self-eliminate. Yet, we find that DNA damaging drugs are still generally effective against cancer cells. One possibility is that in these cells, DNA damaging drugs are simultaneously inducing mtDNA damage alongside DNA damage. That is not to say that the effects of DNA damage are inconsequential, but instead that there may be a synergistic effect in many DNA damaging chemotherapies between mtDNA damage with nuclear DNA damage. Future studies should be conducted to analyze the relative contributions of mtDNA versus nuclear DNA damage and how each contributes to cellular degeneration when the entire cell is exposed to DNA damage.

4.3 Future directions

Cisplatin and other DNA damaging drugs

While our findings have yielded interesting results, there remains a number of important questions that need to be answered. Cisplatin-induced CIPN and the axon degeneration associated with it remains a problem, but cisplatin has been implicated in other non-nucleoside binding events (75). These non-DNA bind events have included a number of proteins which if bound by cisplatin could lead to direct negative consequences (225). While these complications do not diminish the value and insight of our findings as it relates to cisplatin-induced CIPN, it does raise a flag of caution. That concern is why we confirmed our findings that both d4T and camptothecin were capable of inducing axonal degeneration when exposed to only the axons and axonal mitochondria.

Cisplatin mediates its DNA damage by binding to DNA bases themselves, but other bases within a cell are capable of reacting with its active moiety to cause binding events. Unlike cisplatin, d4T is an antiretroviral nucleoside analog which is capable of being incorporated into the mitochondrial DNA by the mitochondrial polymerase POLG and has be associated with peripheral neuropathies in patients undergoing therapy for HIV infection (129). Once it is attempted to be incorporated into the mtDNA by POLG it leads to the stalling of POLG, which can lead to subsequent damage to the mtDNA (193). Camptothecin on the other hand is a topoisomerase-I inhibitor and can induce double-stranded DNA breaks a s a consequence of topoisomerase stalling (192). Thus, these two DNA damaging drugs induce mtDNA through a completely different mechanism of action than cisplatin and because they do not form direct covalent bonds

with cellular materials, they are less likely to exhibit potential off target effects like those observed in cisplatin exposure.

Therefore, it is reasonable to use the DNA damaging drugs d4T and camptothecin to follow-up our findings with cisplatin in axonal mitochondria. As there might be some concern that our findings could be restricted to being relevant only in cisplatin-induced CIPN or global cisplatin exposure, it makes sense to conduct mechanistic follow-up studies using drugs that more specifically damage mtDNA with less potential off target effects. Our results that d4T and camptothecin are capable of inducing axon degeneration are promising and it would be interesting to examine of the axon degeneration induced by these drugs is mediated by the three pathways of axon degeneration we previously examined in the context of cisplatin.

Mitochondrial depolarization and mitophagy

In Chapter Three, we observed that mitochondria exposed to cisplatin appeared to aggregate, become depolarized, and cease trafficking along microtubules. These data are not fully complete, and these data should be expanded upon to make these findings publishable.

Our results also indicate that cisplatin-damaged mitochondria lose their membrane potential. Mitochondria which lose their membrane potential are targeted for degradation by the mitophagy pathway. One interesting question that remains unanswered is if the mitophagy pathway engaged in response to cisplatin-induced mtDNA damage is protective or damaging to the outcome of the exposure cell. This is an interesting question because there are reasonable arguments for both sides. For

example, if damaged mitochondria are suffering from failure to maintain the proper functionality of the mitochondrial ETC and are producing ROS then they are potentially threatening the viability of the cell. If that ROS production remains unchecked then the ROS could further induce mtDNA damage as well as potentially damage other, previously undamaged, mitochondria and amplify the amount of damage experienced by the cell. In that scenario, it makes sense that the cell would want to engage the mitophagy pathway rapidly to eliminate any compromised mitochondria before they can cause further damage to the cell.

However, on the other hand, if the cellular mitochondria are exposed to a huge impulse of DNA damage which damages almost all of the mitochondria then more caution is beneficial. If almost all the mitochondria of a cell become damaged and subsequently eliminated by mitophagy then it would leave the cell with severely diminished mitochondria and could lead to cellular failure due to mitochondrial loss. In that case, giving mitochondria time to attempt to repair themselves and their mtDNA to reestablish a membrane potential and ward off mitophagy would lead to an increased number of saved mitochondria that would allow a cell to survive.

We propose than examining the consequences of mtDNA damage in Parkin-deficient neurons would provide insight into the role of mitophagy in the context of mtDNA damage. Parkin-deficient neurons are unable to selectively target depolarized mitochondria for degradation and thus are deficient in their ability to undergo mitophagy. Thus, if mitophagy is a pro-survival pathway in the context of mtDNA damage we expect that Parkin-deficiency would lead to reduced axon degeneration. If elimination of damaged mitochondria was a key role of mitophagy in mtDNA damage then we would

expect that Parkin-deficient neurons would undergo axon degeneration more quickly. Either of these possibilities is possible and either one provides an interested insight into how neurons, and cells in general, handle mtDNA damage and attempt to mitigate its effects.

4.4 Concluding remarks

The work described here shows that cisplatin is directly capable of inducing mtDNA and axon degeneration. While we found that this degeneration was not mediated by the known pathways of axon degeneration (apoptosis, axon pruning, and Wallerian degeneration), we did find that mtDNA damage induced axon degeneration was protected against by the inhibition of ROS by GSH. These findings have implications in the current treatment of cisplatin-induced CIPN by patients and the viability of GSH supplementation. In addition, these data shown that axonal mtDNA damage induced by cisplatin leads to a wide variety of mitochondrial deficits and compromised functionality. These findings suggest that mtDNA damage may play an important role in the cellular response to global DNA damage, and provide a more complex response that that induced by nuclear DNA damage alone.

REFERENCES

1. Ernster L, Schatz G. Mitochondria: a historical review. J Cell Biol. 1981;91(3):227s-55s.

2. Antico Arciuch VG, Elguero ME, Poderoso JJ, Carreras MC. Mitochondrial regulation of cell cycle and proliferation. Antioxid Redox Signal. 2012;16(10):1150-80.

3. Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC. Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. J Cell Biol. 2003;160(2):189-200.

4. Wanet A, Arnould T, Najimi M, Renard P. Connecting mitochondria, metabolism, and stem cell fate. Stem Cells Dev. 2015;24(17):1957-71.

5. Rehman J. Empowering self-renewal and differentiation: the role of mitochondria in stem cells. J Mol Med. 2010;88(10):981-6.

6. McBride HM, Neuspiel M, Wasiak S. Mitochondria: more than just a powerhouse. Curr Biol. 2006;16(14):R551-R60.

7. Wang C, Youle RJ. The role of mitochondria in apoptosis. Annu Rev Genet. 2009;43:95-118.

8. Wallin IE. The mitochondria problem. Am Nat. 1923;57(650):255-61.

9. Emelyanov VV. Rickettsiaceae, rickettsia-like endosymbionts, and the origin of mitochondria. Biosci Rep. 2001;21(1):1-17.

10. Gray MW, Burger G, Lang BF. Mitochondrial Evolution. Science. 1999;283(5407):1476-81.

11. Keeling PJ, Archibald JM. Organelle evolution: what's in a name? Curr Biol. 2008;18(8):345-7.

12. Wiemerslage L, Lee D. Quantification of mitochondrial morphology in neurites of dopaminergic neurons using multiple parameters. J Neurosci Methods. 2016;262:56-65.

13. Mannella CA. The ‘ins’ and ‘outs’ of mitochondrial membrane channels. Trends Biochem Sci. 1992;17(8):315-20.

14. Zick M, Rabl R, Reichert AS. Cristae formation—linking ultrastructure and function of mitochondria. Biochim Biophys Acta. 2009;1793(1):5-19.

15. Osman C, Voelker DR, Langer T. Making heads or tails of phospholipids in mitochondria. J Cell Biol. 2011;192(1):7-16.

16. Hatefi Y. The mitochondrial electron transport and oxidative phosphorylation system. Annu Rev Biochem. 1985;54(1):1015-69.

17. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell. 1997;91(4):479-89.

18. Reed JC. Cytochrome c: can't live with it--can't live without it. Cell. 1997;91(5):559-62.

19. Voet D, Voet J, Pratt C. Fundamentals of biochemistry life at the molecular level. New York City: John Wiley & Sons, Inc.; 2013.

20. Schon EA, DiMauro S, Hirano M. Human mitochondrial DNA: roles of inherited and somatic mutations. Nat Rev Genet. 2012;13(12):878-90.

21. Ow Y-LP, Green DR, Hao Z, Mak TW. Cytochrome c: functions beyond respiration. Nat Rev Mol Cell Biol. 2008;9(7):532-42.

22. Chance B, Williams GR. The respiratory chain and oxidative phosphorylation. Advances in Enzymology and Related Areas of Molecular Biology: John Wiley & Sons, Inc.; 2006. p. 65-134.

23. Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. Nature. 1981;290(5806):457-65.

24. Bibb MJ, Van Etten RA, Wright CT, Walberg MW, Clayton DA. Sequence and gene organization of mouse mitochondrial DNA. Cell. 1981;26(2):167-80.

25. Falkenberg M, Larsson N-G, Gustafsson CM. DNA replication and transcription in mammalian mitochondria. Annu Rev Biochem. 2007;76:679-99.

26. Wallace DC, Brown MD, Lott MT. Mitochondrial DNA variation in human evolution and disease. Gene. 1999;238(1):211-30.

27. Calvo SE, Mootha VK. The mitochondrial proteome and human disease. Annu Rev Genomics Hum Genet. 2010;11:25-44.

28. Gaston D, Tsaousis AD, Roger AJ. Chapter 2: Predicting proteomes of mitochondria and related organelles from genomic and expressed sequence tag data. Methods Enzymol. Volume 457: Academic Press; 2009. p. 21-47.

29. Wiesner RJ, Rüegg JC, Morano I. Counting target molecules by exponential polymerase chain reaction: Copy number of mitochondrial DNA in rat tissues. Biochem Biophys Res Comm. 1992;183(2):553-9.

30. Satoh M, Kuroiwa T. Organization of multiple nucleoids and DNA molecules in mitochondria of a human cell. Exp Cell Res. 1991;196(1):137-40.

31. Kang D, Kim SH, Hamasaki N. Mitochondrial transcription factor A (TFAM): Roles in maintenance of mtDNA and cellular functions. Mitochondrion. 2007;7(1–2):39-44.

32. Istiaq Alam T, Kanki T, Muta T, Ukaji K, Abe Y, Nakayama H, et al. Human mitochondrial DNA is packaged with TFAM. Nucleic Acids Res. 2003;31(6):1640-5.

33. Case JT, Wallace DC. Maternal inheritance of mitochondrial DNA polymorphisms in cultured human fibroblasts. Somatic Cell Genet. 1981;7(1):103-8.

34. DeLuca Steven Z, O'Farrell Patrick H. Barriers to male transmission of mitochondrial DNA in sperm development. Dev Cell. 2012;22(3):660-8.

35. Yu Z, O'Farrell PH, Yakubovich N, DeLuca SZ. The mitochondrial DNA polymerase promotes elimination of paternal mitochondrial genomes. Curr Biol. 2017;27(7):1033-9.

36. Nishimura Y, Yoshinari T, Naruse K, Yamada T, Sumi K, Mitani H, et al. Active digestion of sperm mitochondrial DNA in single living sperm revealed by optical tweezers. Proc Nat Acad Sci USA. 2006;103(5):1382-7.

37. Sato M, Sato K. Maternal inheritance of mitochondrial DNA by diverse mechanisms to eliminate paternal mitochondrial DNA. Biochim Biophys Acta. 2013;1833(8):1979-84.

38. Sutovsky P, Moreno RD, Ramalho-Santos J, Dominko T, Simerly C, Schatten G. Development: Ubiquitin tag for sperm mitochondria. Nature. 1999;402(6760):371-2.

39. Chan DC, Schon EA. Eliminating mitochondrial DNA from sperm. Dev Cell. 2012;22(3):469-70.

40. St John J, Sakkas D, Dimitriadi K, Barnes A, Maclin V, Ramey J, et al. Failure of elimination of paternal mitochondrial DNA in abnormal embryos. Lancet. 2000;355(9199):200.

41. Tuppen HAL, Blakely EL, Turnbull DM, Taylor RW. Mitochondrial DNA mutations and human disease. Biochim Biophys Acta. 2010;1797(2):113-28.

42. Chan DC. Mitochondrial fusion and fission in mammals. Annu Rev Cell Dev Biol. 2006;22:79-99.

43. Chan DC. Fusion and fission: interlinked processes critical for mitochondrial health. Annu Rev Genet. 2012.

44. Youle RJ, van der Bliek AM. Mitochondrial fission, fusion, and stress. Science. 2012;337(6098):1062-5.

45. Akhmedov AT, Marín-García J. Mitochondrial DNA maintenance: an appraisal. Mol Cell Biochem. 2015;409(1):283-305.

46. Pohjoismäki JLO, Goffart S, Tyynismaa H, Willcox S, Ide T, Kang D, et al. Human heart mitochondrial DNA is organized in complex catenated networks containing abundant four-way junctions and replication forks. J Biol Chem. 2009;284(32):21446-57.

47. Mengel-From J, Thinggaard M, Dalgård C, Kyvik KO, Christensen K, Christiansen L. Mitochondrial DNA copy number in peripheral blood cells declines with age and is associated with general health among elderly. Hum Genet. 2014;133(9):1149-59.

48. Barazzoni R, Short KR, Nair KS. Effects of aging on mitochondrial DNA copy number and cytochrome c oxidase gene expression in rat skeletal muscle, liver, and heart. J Biol Chem. 2000;275(5):3343-7.

49. Hu L, Yao X, Shen Y. Altered mitochondrial DNA copy number contributes to human cancer risk: evidence from an updated meta-analysis. Sci Rep. 2016;6:35859.

50. Pyle A, Anugrha H, Kurzawa-Akanbi M, Yarnall A, Burn D, Hudson G. Reduced mitochondrial DNA copy number is a biomarker of Parkinson's disease. Neurobiol Aging. 2016;38:216.e7-.e10.

51. Stumpf JD, Saneto RP, Copeland WC. Clinical and molecular features of POLG-related mitochondrial disease. Cold Spring Harb Perspect Biol. 2013;5(4):a011395.

52. Sarzi E, Goffart S, Serre V, Chrétien D, Slama A, Munnich A, et al. Twinkle helicase (PEO1) gene mutation causes mitochondrial DNA depletion. Annals Neurol. 2007;62(6):579-87.

53. McKinney EA, Oliveira MT. Replicating animal mitochondrial DNA. Genet Mol Biol. 2013;36(3):308-15.

54. Taanman J-W. The mitochondrial genome: structure, transcription, translation and replication. Biochim Biophys Acta. 1999;1410(2):103-23.

55. Ekstrand MI, Falkenberg M, Rantanen A, Park CB, Gaspari M, Hultenby K, et al. Mitochondrial transcription factor A regulates mtDNA copy number in mammals. Hum Mol Genet. 2004;13(9):935-44.

56. Larsson N-G, Wang J, Wilhelmsson H, Oldfors A, Rustin P, Lewandoski M, et al. Mitochondrial transcription factor A is necessary for mtDNA maintance and embryogenesis in mice. Nat Genet. 1998;18(3):231-6.

57. Alexeyev M, Shokolenko I, Wilson G, LeDoux S. The maintenance of mitochondrial DNA integrity—critical analysis and update. Cold Spring Harb Perspect Biol. 2013;5(5).

58. Herbst A, Pak JW, McKenzie D, Bua E, Bassiouni M, Aiken JM. Accumulation of mitochondrial DNA deletion mutations in aged muscle fibers: evidence for a causal role in muscle fiber loss. J Gerontol A Biol Sci Med Sci. 2007;62(3):235-45.

59. Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, et al. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat Genet. 2006;38(5):515-7.

60. Chatterjee A, Mambo E, Sidransky D. Mitochondrial DNA mutations in human cancer. Oncogene. 0000;25(34):4663-74.

61. Inoue K, Nakada K, Ogura A, Isobe K, Goto Y-i, Nonaka I, et al. Generation of mice with mitochondrial dysfunction by introducing mouse mtDNA carrying a deletion into zygotes. Nat Genet. 2000;26(2):176-81.

62. Holt IJ, Harding AE, Morgan-Hughes JA. Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. Nature. 1988;331(6158):717-9.

63. Moraes CT, DiMauro S, Zeviani M, Lombes A, Shanske S, Miranda AF, et al. Mitochondrial DNA deletions in progressive external ophthalmoplegia and Kearns-Sayre syndrome. New Eng J Med. 1989;320(20):1293-9.

64. Lee HR, Johnson KA. Fidelity of the human mitochondrial DNA polymerase. J Biol Chem. 2006;281(47):36236-40.

65. Cline SD. Mitochondrial DNA damage and its consequences for mitochondrial gene expression. Biochim Biophys Acta. 2012;1819(9-10):979-91.

66. Yakes FM, Van Houten B. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. Proc Natl Acad Sci USA. 1997;94(2):514-9.

67. De Bont R, van Larebeke N. Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis. 2004;19(3):169-85.

68. Hanes JW, Thal DM, Johnson KA. Incorporation and replication of 8-Oxo-deoxyguanosine by the human pitochondrial DNA polymerase. J Biol Chem. 2006;281(47):36241-8.

69. Pinz KG, Bogenhagen DF. Efficient repair of abasic sites in DNA by mitochondrial enzymes. Mol Cell Biol. 1998;18(3):1257-65.

70. Lim SE, Copeland WC. Differential incorporation and removal of antiviral deoxynucleotides by human DNA polymerase γ. J Biol Chem. 2001;276(26):23616-23.

71. Rydberg B, Lindahl T. Nonenzymatic methylation of DNA by the intracellular methyl group donor S-adenosyl-L-methionine is a potentially mutagenic reaction. EMBO J. 1982;1(2):211-6.

72. LeDoux SP, Driggers WJ, Hollensworth BS, Wilson GL. Repair of alkylation and oxidative damage in mitochondrial DNA. Mutat Res. 1999;434(3):149-59.

73. Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. Nat Rev Drug Discov. 2005;4(4):307-20.

74. Pinto AL, Lippard SJ. Sequence-dependent termination of in vitro DNA synthesis by cis- and trans-diamminedichloroplatinum (II). Proc Nat Acad Sci USA. 1985;82(14):4616-9.

75. Todd RC, Lippard SJ. Consequences of cisplatin binding on nucleosome structure and dynamics. Chem Biol. 2010;17(12):1334-43.

76. Svilar D, Goellner EM, Almeida KH, Sobol RW. Base excision repair and lesion-dependent subpathways for repair of oxidative DNA damage. Antiox Redox Signal. 2011;14(12):2491-507.

77. Thorslund T, Sunesen M, Bohr VA, Stevnsner T. Repair of 8-oxoG is slower in endogenous nuclear genes than in mitochondrial DNA and is without strand bias. DNA repair. 2002;1(4):261-73.

78. Szczesny B, Tann AW, Longley MJ, Copeland WC, Mitra S. Long patch base excision repair in mammalian mitochondrial genomes. J Biol Chem. 2008;283(39):26349-56.

79. Akbari M, Visnes T, Krokan HE, Otterlei M. Mitochondrial base excision repair of uracil and AP sites takes place by single-nucleotide insertion and long-patch DNA synthesis. DNA Repair. 2008;7(4):605-16.

80. Croteau DL, Bohr VA. Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells. J Biol Chem. 1997;272(41):25409-12.

81. Boesch P, Weber-Lotfi F, Ibrahim N, Tarasenko V, Cosset A, Paulus F, et al. DNA repair in organelles: pathways, organization, regulation, relevance in disease and aging. Biochim Biophys Acta. 2011;1813(1):186-200.

82. LeDoux SP, Wilson GL, Beecham EJ, Stevnsner T, Wassermann K, Bohr VA. Repair of mitochondrial DNA after various types of DNA damage in Chinese hamster ovary cells. Carcinogenesis. 1992;13(11):1967-73.

83. Clayton DA, Doda JN, Friedberg EC. The absence of a pyrimidine dimer mepair mechanism in mammalian mitochondria. Proc Nat Acad Sci USA. 1974;71(7):2777-81.

84. Pascucci B, Versteegh A, van Hoffen A, van Zeeland AA, Mullenders LHF, Dogliotti E. DNA repair of UV photoproducts and mutagenesis in human mitochondrial DNA1. J Mol Biol. 1997;273(2):417-27.

85. Copeland WC, Longley MJ. Mitochondrial genome maintenance in health and disease. DNA Repair. 2014;19:190-8.

86. Mason PA, Matheson EC, Hall AG, Lightowlers RN. Mismatch repair activity in mammalian mitochondria. Nuc Acids Res. 2003;31(3):1052-8.

87. de Souza-Pinto NC, Mason PA, Hashiguchi K, Weissman L, Tian J, Guay D, et al. Novel DNA mismatch repair activity involving YB-1 in human mitochondria. DNA repair. 2009;8(6):704-19.

88. Zhou J, Liu L, Chen J. Mitochondrial DNA heteroplasmy in Candida glabrata after mitochondrial transformation. Eukaryot Cell. 2010;9(5):806-14.

89. Mileshina D, Koulintchenko M, Konstantinov Y, Dietrich A. Transfection of plant mitochondria and in organello gene integration. Nucleic Acids Res. 2011;39(17):e115-e.

90. Manchekar M, Scissum-Gunn K, Song D, Khazi F, McLean SL, Nielsen BL. DNA recombination activity in soybean mitochondria. J Mol Biol. 2006;356(2):288-99.

91. Gilkerson RW, Schon EA, Hernandez E, Davidson MM. Mitochondrial nucleoids maintain genetic autonomy but allow for functional complementation. J Cell Biol. 2008;181(7):1117-28.

92. Lakshmipathy U, Campbell C. Double strand break rejoining by mammalian mitochondrial extracts. Nucleic Acids Res. 1999;27(4):1198-204.

93. Youle RJ, Narendra DP. Mechanisms of mitophagy. Nat Rev Mol Cell Biol. 2011;12(1):9-14.

94. Narendra DP, Jin SM, Tanaka A, Suen D-F, Gautier CA, Shen J, et al. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol. 2010;8(1):e1000298.

95. Matsuda N, Sato S, Shiba K, Okatsu K, Saisho K, Gautier CA, et al. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. J Cell Biol. 2010;189(2):211-21.

96. Lin W, Kang UJ. Characterization of PINK1 processing, stability, and subcellular localization. J Neurochem. 2008;106(1):464-74.

97. Kane LA, Lazarou M, Fogel AI, Li Y, Yamano K, Sarraf SA, et al. PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity. J Cell Biol. 2014;205(2):143-53.

98. Ashrafi G, Schwarz TL. The pathways of mitophagy for quality control and clearance of mitochondria. Cell Death Differ. 2013;20(1):31-42.

99. Nguyen TN, Padman BS, Lazarou M. Deciphering the molecular signals of PINK1/Parkin mitophagy. Trends Cell Biol. 2016;26(10):733-44.

100. Murphy Michael P. How mitochondria produce reactive oxygen species. Biochem J. 2009;417(Pt 1):1-13.

101. Fukai T, Ushio-Fukai M. Superoxide dismutases: role in redox signaling, vascular function, and diseases. Antioxid Redox Signal. 2011;15(6):1583-606.

102. Cadenas E, Davies KJA. Mitochondrial free radical generation, oxidative stress, and aging. Free Rad Biol Med. 2000;29(3–4):222-30.

103. D'Autreaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nat Rev Mol Cell Biol. 2007;8(10):813-24.

104. Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol. 2000;279(6):L1005-L28.

105. Mikhed Y, Daiber A, Steven S. Mitochondrial oxidative stress, mitochondrial DNA damage and their role in age-related vascular dysfunction. Int J Mol Sci. 2015;16(7):15918-53.

106. Marullo R, Werner E, Degtyareva N, Moore B, Altavilla G, Ramalingam SS, et al. Cisplatin induces a mitochondrial-ROS response that contributes to cytotoxicity depending on mitochondrial redox status and bioenergetic functions. PLoS ONE. 2013;8(11):e81162.

107. Marí M, Morales A, Colell A, García-Ruiz C, Fernández-Checa JC. Mitochondrial glutathione, a key survival antioxidant. Antioxid Redox Signal. 2009;11(11):2685-700.

108. Deneke SM, Fanburg BL. Regulation of cellular glutathione. Am J Physiol Lung Cell Mol Physiol. 1989;257(4):L163-L73.

109. Lewis MR, Lewis WH. Mitochondria (and other cytoplasmic structures) in tissue cultures. Dev Dyn. 1915;17(3):339-401.

110. Bereiter-Hahn J, Vöth M. Dynamics of mitochondria in living cells: shape changes, dislocations, fusion, and fission of mitochondria. Microsc Res Tech. 1994;27(3):198-219.

111. Rizzuto R, Brini M, De Giorgi F, Rossi R, Heim R, Tsien RY, et al. Double labelling of subcellular structures with organelle-targeted GFP mutants in vivo. Curr Biol. 1996;6(2):183-8.

112. Karbowski M. Mitochondria on guard: role of mitochondrial fusion and fission in the regulation of apoptosis. Adv Exp Med Biol. 2010;687:131-42.

113. Chen H, Chan DC. Emerging functions of mammalian mitochondrial fusion and fission. Hum Mol Genet. 2005;14 Spec No. 2:R283-9.

114. Hoppins S, Lackner L, Nunnari J. The machines that divide and fuse mitochondria. Annu Rev Biochem. 2007;76:751-80.

115. Hoppins S, Edlich F, Cleland MM, Banerjee S, McCaffery JM, Youle RJ, et al. The soluble form of Bax regulates mitochondrial fusion via MFN2 homotypic complexes. Mol Cell. 2011;41(2):150-60.

116. Debanne D, Campanac E, Bialowas A, Carlier E, Alcaraz G. Axon Physiology. Physiol Rev. 2011;91(2):555-602.

117. Ligon LA, Steward O. Role of microtubules and actin filaments in the movement of mitochondria in the axons and dendrites of cultured hippocampal neurons. J Comp Neurol. 2000;427(3):351-61.

118. Hollenbeck PJ, Saxton WM. The axonal transport of mitochondria. J Cell Sci. 2005;118(23):5411-9.

119. Misko A, Jiang S, Wegorzewska I, Milbrandt J, Baloh RH. Mitofusin 2 is necessary for transport of axonal mitochondria and interacts with the Miro/Milton complex. J Neurosci. 2010;30(12):4232-40.

120. Lovas JR, Wang X. The meaning of mitochondrial movement to a neuron's life. Biochim Biophys Acta. 2013;1833(1):184-94.

121. Wang X, Winter D, Ashrafi G, Schlehe J, Wong Yao L, Selkoe D, et al. PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. Cell.147(4):893-906.

122. Windebank AJ, Grisold W. Chemotherapy-induced neuropathy. J Periph Nerv Sys. 2008;13(1):27-46.

123. Quasthoff S, Hartung HP. Chemotherapy-induced peripheral neuropathy. J Neurol. 2002;249(1):9-17.

124. Wolf S, Barton D, Kottschade L, Grothey A, Loprinzi C. Chemotherapy-induced peripheral neuropathy: Prevention and treatment strategies. Eur J Cancer. 2008;44(11):1507-15.

125. Park SB, Goldstein D, Krishnan AV, Lin CSY, Friedlander ML, Cassidy J, et al. Chemotherapy-induced peripheral neurotoxicity: A critical analysis. CA Cancer J Clin. 2013;63(6):419-37.

126. Gutiérrez-Gutiérrez G, Sereno M, Miralles A, Casado-Sáenz E, Gutiérrez-Rivas E. Chemotherapy-induced peripheral neuropathy: clinical features, diagnosis, prevention and treatment strategies. Clin Transl Oncol. 2010;12(2):81-91.

127. Argyriou AA, Bruna J, Marmiroli P, Cavaletti G. Chemotherapy-induced peripheral neurotoxicity (CIPN): An update. Cr Rev Oncol-Hem. 2012;82(1):51-77.

128. Grisold W, Cavaletti G, Windebank AJ. Peripheral neuropathies from chemotherapeutics and targeted agents: diagnosis, treatment, and prevention. Neuro Oncol. 2012;14(suppl 4):iv45-iv54.

129. Dalakas MC. Peripheral neuropathy and antiretroviral drugs. J Periph Nerv Sys. 2001;6(1):14-20.

130. Staff NP, Grisold A, Grisold W, Windebank AJ. Chemotherapy-induced peripheral neuropathy: a current review. Annals Neurol. 2017.

131. Peters CM, Jimenez-Andrade JM, Kuskowski MA, Ghilardi JR, Mantyh PW. An evolving cellular pathology occurs in dorsal root ganglia, peripheral nerve and spinal cord following intravenous administration of paclitaxel in the rat. Brain Res. 2007;1168:46-59.

132. Topp KS, Tanner KD, Levine JD. Damage to the cytoskeleton of large diameter sensory neurons and myelinated axons in vincristine-induced painful peripheral neuropathy in the rat. J Comp Neurol. 2000;424(4):563-76.

133. Gregg RW, Molepo JM, Monpetit VJ, Mikael NZ, Redmond D, Gadia M, et al. Cisplatin neurotoxicity: the relationship between dosage, time, and platinum concentration in neurologic tissues, and morphologic evidence of toxicity. J Clin Oncol. 1992;10(5):795-803.

134. Cersosimo RJ. Oxaliplatin-associated neuropathy: a review. Annals Pharmacother. 2005;39(1):128-35.

135. McDonald ES, Windebank AJ. Cisplatin-Induced Apoptosis of DRG Neurons Involves Bax Redistribution and Cytochrome c Release But Not fas Receptor Signaling. Neurobiol Dis. 2002;9(2):220-33.

136. Podratz JL, Knight AM, Ta LE, Staff NP, Gass JM, Genelin K, et al. Cisplatin induced mitochondrial DNA damage in dorsal root ganglion neurons. Neurobiol Dis. 2011;41(3):661-8.

137. Ta LE, Espeset L, Podratz J, Windebank AJ. Neurotoxicity of oxaliplatin and cisplatin for dorsal root ganglion neurons correlates with platinum–DNA binding. NeuroToxicology. 2006;27(6):992-1002.

138. Riccomagno MM, Kolodkin AL. Sculpting neural circuits by axon and dendrite pruning. Annu Rev Cell Dev Biol. 2015;31:779-805.

139. Conforti L, Gilley J, Coleman MP. Wallerian degeneration: an emerging axon death pathway linking injury and disease. Nat Rev Neurosci. 2014;15(6):394-409.

140. Mack TGA, Reiner M, Beirowski B, Mi W, Emanuelli M, Wagner D, et al. Wallerian degeneration of injured axons and synapses is delayed by a Ube4b/Nmnat chimeric gene. Nat Neurosci. 2001;4(12):1199-206.

141. Wang J, Zhai Q, Chen Y, Lin E, Gu W, McBurney MW, et al. A local mechanism mediates NAD-dependent protection of axon degeneration. J Cell Biol. 2005;170(3):349-55.

142. Araki T, Sasaki Y, Milbrandt J. Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. Science. 2004;305(5686):1010-3.

143. Wang JT, Medress ZA, Vargas ME, Barres BA. Local axonal protection by WldS as revealed by conditional regulation of protein stability. Proc Natl Acad Sci. 2015;112(33):10093-100.

144. Di Stefano M, Nascimento-Ferreira I, Orsomando G, Mori V, Gilley J, Brown R, et al. A rise in NAD precursor nicotinamide mononucleotide (NMN) after injury promotes axon degeneration. Cell Death Differ. 2015;22(5):731-42.

145. Osterloh JM, Yang J, Rooney TM, Fox AN, Adalbert R, Powell EH, et al. dSarm/Sarm1 is required for activation of an injury-induced axon death pathway. Science. 2012;337:481-4.

146. Gerdts J, Summers DW, Sasaki Y, DiAntonio A, Milbrandt J. Sarm1-mediated axon degeneration requires both SAM and TIR interactions. J Neurosci. 2013;33(33):13569-80.

147. Gerdts J, Brace EJ, Sasaki Y, DiAntonio A, Milbrandt J. SARM1 activation triggers axon degeneration locally via NAD+ destruction. Science. 2015;348(6233):453-7.

148. Gilley J, Coleman MP. Endogenous Nmnat2 Is an Essential Survival Factor for Maintenance of Healthy Axons. PLoS Biol. 2010;8(1):e1000300.

149. Gilley J, Orsomando G, Nascimento-Ferreira I, Coleman Michael P. Absence of SARM1 Rescues Development and Survival of NMNAT2-Deficient Axons. Cell Rep. 2015;10(12):1974-81.

150. Yang J, Wu Z, Renier N, Simon David J, Uryu K, Park David S, et al. Pathological axonal death through a MAPK cascade that triggers a local energy deficit. Cell. 2015;160(1–2):161-76.

151. Miller BR, Press C, Daniels RW, Sasaki Y, Milbrandt J, DiAntonio A. A dual leucine kinase-dependent axon self-destruction program promotes Wallerian degeneration. Nat Neurosci. 2009;12(4):387-9.

152. Vargas ME, Yamagishi Y, Tessier-Lavigne M, Sagasti A. Live imaging of calcium dynamics during axon degeneration reveals two functionally distinct phases of calcium influx. J Neurosci. 2015;35(45):15026-38.

153. Yang J, Weimer Robby M, Kallop D, Olsen O, Wu Z, Renier N, et al. Regulation of axon degeneration after injury and in development by the endogenous calpain inhibitor calpastatin. Neuron. 2013;80(5):1175-89.

154. Loreto A, Di Stefano M, Gering M, Conforti L. Wallerian degeneration is executed by an NMN-SARM1-dependent late Ca2+ influx but only modestly influenced by mitochondria. Cell Rep. 2015;13(11):2539-52.

155. Buss RR, Sun W, Oppenheim RW. Adaptive roles of programmed cell death during nervous system development. Annu Rev Neurosci. 2006;29:1-35.

156. Kristiansen M, Ham J. Programmed cell death during neuronal development: the sympathetic neuron model. Cell Death Differ. 2014;21:1025-35.

157. Glebova NO, Ginty DD. Growth and survival signals controlling sympathetic nervous system development. Annu Rev Neurosci. 2005;28:191-222.

158. Eichler ME, Rich KM. Death of sensory ganglion neurons after acute withdrawal of nerve growth factor in dissociated cell cultures. Brain Res. 1989;482(2):340-6.

159. Simon David J, Pitts J, Hertz Nicholas T, Yang J, Yamagishi Y, Olsen O, et al. Axon degeneration gated by retrograde activation of somatic pro-apoptotic signaling. Cell. 2016;164(5):1031-45.

160. Walensky LD, Gavathiotis E. BAX unleashed: the biochemical transformation of an inactive cytosolic monomer into a toxic mitochondrial pore. Trends Biochem Sci. 2011;36:642-52.

161. Courchesne SL, Karch C, Pazyra-Murphy MF, Segal RA. Sensory neuropathy attributable to loss of Bcl-w. J Neurosci. 2011;31(5):1624-34.

162. Wang X. The expanding role of mitochondria in apoptosis. Genes Dev. 2001;15(22):2922-33.

163. Wright KM, Vaughn AE, Deshmukh M. Apoptosome dependent caspase-3 activation pathway is non-redundant and necessary for apoptosis in sympathetic neurons. Cell Death Differ. 2007;14:625-33.

164. Potts PR, Singh S, Knezek M, Thompson CB, Deshmukh M. Critical function of endogenous XIAP in regulating caspase activation during sympathetic neuronal apoptosis. J Cell Biol. 2003;163:789-99.

165. Unsain N, Higgins Julia M, Parker Kristen N, Johnstone Aaron D, Barker Philip A. XIAP regulates caspase activity in degenerating axons. Cell Rep. 2013;4(0):751-63.

166. Ghosh AS, Wang B, Pozniak CD, Chen M, Watts RJ, Lewcock JW. DLK induces developmental neuronal degeneration via selective regulation of proapoptotic JNK activity. J Cell Biol. 2011;194(5):751-64.

167. Luo L, O'Leary DD. Axon retraction and degeneration in development and disease. Annu Rev Neurosci. 2005;28:127-56.

168. Campenot RB. Local control of neurite development by nerve growth factor. Proc Natl Acad Sci USA. 1977;74(10):4516-9.

169. Taylor AM, Blurton-Jones M, Rhee SW, Cribbs DH, Cotman CW, Jeon NL. A microfluidic culture platform for CNS axonal injury, regeneration and transport. Nat Methods. 2005;2(8):599-605.

170. Nikolaev A, McLaughlin T, O'Leary DD, Tessier-Lavigne M. APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. Nature. 2009;457(7232):981-9.

171. Olsen O, Kallop DY, McLaughlin T, Huntwork-Rodriguez S, Wu Z, Duggan CD, et al. Genetic analysis reveals that amyloid precursor protein and death receptor 6 function in the same pathway to control axonal pruning independent of β-secretase. J Neurosci. 2014;34(19):6438-47.

172. Chen M, Maloney JA, Kallop DY, Atwal JK, Tam SJ, Baer K, et al. Spatially coordinated kinase signaling regulates local axon degeneration. J Neurosci. 2012;32(39):13439-53.

173. Mok SA, Lund K, Campenot RB. A retrograde apoptotic signal originating in NGF-deprived distal axons of rat sympathetic neurons in compartmented cultures. Cell Res. 2009;19(5):546-60.

174. Cusack CL, Swahari V, Hampton Henley W, Michael Ramsey J, Deshmukh M. Distinct pathways mediate axon degeneration during apoptosis and axon-specific pruning. Nat Commun. 2013;4:1876.

175. Schoenmann Z, Assa-Kunik E, Tiomny S, Minis A, Haklai-Topper L, Arama E, et al. Axonal degeneration is regulated by the apoptotic machinery or a NAD+-sensitive pathway in insects and mammals. J Neurosci. 2010;30(18):6375-86.

176. Simon DJ, Weimer RM, McLaughlin T, Kallop D, Stanger K, Yang J, et al. A caspase cascade regulating developmental axon degeneration. J Neurosci. 2012;32(49):17540-53.

177. Wright KM, Smith MI, Farrag L, Deshmukh M. Chromatin modification of Apaf-1 restricts the apoptotic pathway in mature neurons. J Cell Biol. 2007;179(5):825-32.

178. Unsain N, Barker Philip A. New views on the misconstrued: executioner caspases and their diverse non-apoptotic roles. Neuron. 2015;88(3):461-74.

179. Kuo CT, Zhu S, Younger S, Jan LY, Jan YN. Identification of E2/E3 ubiquitinating enzymes and caspase activity regulating Drosophila sensory neuron dendrite pruning. Neuron. 2006;51(3):283-90.

180. Jeggo PA, Pearl LH, Carr AM. DNA repair, genome stability and cancer: a historical perspective. Nat Rev Cancer. 2016;16(1):35-42.

181. Roos WP, Thomas AD, Kaina B. DNA damage and the balance between survival and death in cancer biology. Nat Rev Cancer. 2016;16(1):20-33.

182. Ciccia A, Elledge SJ. The DNA damage response: making it safe to play with knives. Mol Cell. 2010;40(2):179-204.

183. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. Cell. 2002;108(2):153-64.

184. Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, et al. Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature. 2004;429(6990):417-23.

185. Kujoth GC, Hiona A, Pugh TD, Someya S, Panzer K, Wohlgemuth SE, et al. Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. Science. 2005;309(5733):481-4.

186. Someya S, Yamasoba T, Kujoth GC, Pugh TD, Weindruch R, Tanokura M, et al. The role of mtDNA mutations in the pathogenesis of age-related hearing loss in mice carrying a mutator DNA polymerase γ. Neurobiol Aging. 2008;29(7):1080-92.

187. Kolesar JE, Safdar A, Abadi A, MacNeil LG, Crane JD, Tarnopolsky MA, et al. Defects in mitochondrial DNA replication and oxidative damage in muscle of mtDNA mutator mice. Free Rad Biol Med. 2014;75:241-51.

188. Tann AW, Boldogh I, Meiss G, Qian W, Van Houten B, Mitra S, et al. Apoptosis induced by persistent single-strand breaks in mitochondrial genome: critical role

of EXOG (5′-exo/endonuclease) in their repair. J Biol Chem. 2011;286(37):31975-83.

189. Park JW, Vahidi B, Taylor AM, Rhee SW, Jeon NL. Microfluidic culture platform for neuroscience research. Nat Protoc. 2006;1(4):2128-36.

190. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem. 1998;273(10):5858-68.

191. Sasaki Y, Vohra BPS, Lund FE, Milbrandt J. Nicotinamide mononucleotide adenylyl transferase-mediated axonal protection requires enzymatic activity but not increased levels of neuronal nicotinamide adenine dinucleotide. J Neurosci. 2009;29(17):5525-35.

192. Wall ME, Wani MC. Camptothecin and taxol: from discovery to clinic. J Ethnopharmacol. 1996;51(1):239-54.

193. Balzarini J. Metabolism and mechanism of antiretroviral action of purine and pyrimidine derivatives. Pharma World Sci. 1994;16(2):113-26.

194. Wyttenbach A, Tolkovsky AM. The BH3-only protein Puma is both necessary and sufficient for neuronal apoptosis induced by DNA damage in sympathetic neurons. J Neurochem. 2006;96(5):1213-26.

195. Vaughn AE, Deshmukh M. Essential postmitochondrial function of p53 uncovered in DNA damage-induced apoptosis in neurons. Cell Death Differ. 2007;14(5):973-81.

196. Mukhopadhyay P, Horváth B, Zsengellér Z, Zielonka J, Tanchian G, Holovac E, et al. Mitochondrial-targeted antioxidants represent a promising approach for prevention of cisplatin-induced nephropathy. Free Rad Biol Med. 2012;52(2):497-506.

197. Tredici G, Cavaletti G, Petruccioli M, Fabbrica D, Tedeschi M, Venturino P. Low-dose glutathione administration in the prevention of cisplatin-induced peripheral neuropathy in rats. Neurotoxicol. 1993;15(3):701-4.

198. Cascinu S, Cordella L, Ferro ED, Fronzoni M, Catalano G. Neuroprotective effect of reduced glutathione on cisplatin-based chemotherapy in advanced gastric cancer: a randomized double-blind placebo-controlled trial. J Clin Oncol. 1995;13(1):26-32.

199. Albers JW, Chaudhry V, Cavaletti G, Donehower RC. Interventions for preventing neuropathy caused by cisplatin and related compounds. The Cochrane Library. 2011.

200. Hashiguchi K, Bohr VA, de Souza-Pinto NC. Oxidative stress and mitochondrial DNA repair: implications for NRTIs induced DNA damage. Mitochondrion. 2004;4(2–3):215-22.

201. Cullen K, Yang Z, Schumaker L, Guo Z. Mitochondria as a critical target of the chemotheraputic agent cisplatin in head and neck cancer. J Bioenerg Biomemb. 2007;39(1):43-50.

202. Olivero OA, Chang PK, Lopez-Larraza DM, Cristina Semino-Mora M, Poirier MC. Preferential formation and decreased removal of cisplatin–DNA adducts in Chinese hamster ovary cell mitochondrial DNA as compared to nuclear DNA. Mut Res/Genet Toxicol Environ Mut. 1997;391(1–2):79-86.

203. Clark-Matott J, Saleem A, Dai Y, Shurubor Y, Ma X, Safdar A, et al. Metabolomic analysis of exercise effects in the POLG mitochondrial DNA mutator mouse brain. Neurobiol Aging. 2015;36(11):2972-83.

204. Safdar A, Khrapko K, Flynn JM, Saleem A, De Lisio M, Johnston AP, et al. Exercise-induced mitochondrial p53 repairs mtDNA mutations in mutator mice. Skelet Muscle. 2016;6:7.

205. Bakhanashvili M, Grinberg S, Bonda E, Simon AJ, Moshitch-Moshkovitz S, Rahav G. p53 in mitochondria enhances the accuracy of DNA synthesis. Cell Death Differ. 2008;15.

206. Achanta G, Sasaki R, Feng L, Carew JS, Lu W, Pelicano H, et al. Novel role of p53 in maintaining mitochondrial genetic stability through interaction with DNA Pol γ. EMBO J. 2005;24(19):3482-92.

207. de Souza-Pinto NC, Harris CC, Bohr VA. p53 functions in the incorporation step in DNA base excision repair in mouse liver mitochondria. Oncogene. 2004;23(39):6559-68.

208. Lebedeva MA, Eaton JS, Shadel GS. Loss of p53 causes mitochondrial DNA depletion and altered mitochondrial reactive oxygen species homeostasis. Biochim Biophys Acta. 2009;1787(5):328-34.

209. Fukui H, Moraes CT. Mechanisms of formation and accumulation of mitochondrial DNA deletions in aging neurons. Human Mol Genet. 2009;18(6):1028-36.

210. Srivastava S, Moraes CT. Double-strand breaks of mouse muscle mtDNA promote large deletions similar to multiple mtDNA deletions in humans. Human Mol Genet. 2005;14(7):893-902.

211. Wang X, Pickrell AM, Rossi SG, Pinto M, Dillon LM, Hida A, et al. Transient systemic mtDNA damage leads to muscle wasting by reducing the satellite cell pool. Human Mol Genet. 2013;22(19):3976-86.

212. Pinto M, Pickrell AM, Wang X, Bacman SR, Yu A, Hida A, et al. Transient mitochondrial DNA double strand breaks in mice cause accelerated aging phenotypes in a ROS-dependent but p53/p21-independent manner. Cell Death Differ. 2017;24(2):288-99.

213. Chamberlain GR, Tulumello DV, Kelley SO. Targeted delivery of doxorubicin to mitochondria. ACS chemical biology. 2013;8(7):1389-95.

214. Wisnovsky Simon P, Wilson Justin J, Radford Robert J, Pereira Mark P, Chan Maria R, Laposa Rebecca R, et al. Targeting mitochondrial DNA with a platinum-based anticancer agent. Chem Biol. 2013;20(11):1323-8.

215. Santos JH, Meyer JN, Mandavilli BS, Van Houten B. Quantitative PCR-based measurement of nuclear and mitochondrial DNA damage and repair in mammalian cells. Methods Mol Biol. 2006;314:183-99.

216. Hunter SE, Jung D, Di Giulio RT, Meyer JN. The QPCR assay for analysis of mitochondrial DNA damage, repair, and relative copy number. Methods. 2010;51(4):444-51.

217. Van Houten B, Hunter SE, Meyer JN. Mitochondrial DNA damage induced autophagy, cell death, and disease. Front Biosci. 2016;21:42-54.

218. Ashrafi G, Schlehe JS, LaVoie MJ, Schwarz TL. Mitophagy of damaged mitochondria occurs locally in distal neuronal axons and requires PINK1 and Parkin. J Cell Biol. 2014;206(5):655-70.

219. Santos JH, Hunakova Lu, Chen Y, Bortner C, Van Houten B. Cell sorting experiments link persistent mitochondrial DNA damage with loss of mitochondrial membrane potential and apoptotic cell death. J Biol Chem. 2003;278(3):1728-34.

220. Lewis SC, Uchiyama LF, Nunnari J. ER-mitochondria contacts couple mtDNA synthesis with mitochondrial division in human cells. Science. 2016;353(6296).

221. Duchen MR. Mitochondria in health and disease: perspectives on a new mitochondrial biology. Mol Aspect Med. 2004;25(4):365-451.

222. Han Y, Smith MT. Pathobiology of cancer chemotherapy-induced peripheral neuropathy (CIPN). Frontiers Pharm. 2013;4.

223. Miltenburg NC, Boogerd W. Chemotherapy-induced neuropathy: A comprehensive survey. Cancer Treat Rev. 2014;40(7):872-82.

224. Cascinu S, Catalano V, Cordella L, Labianca R, Giordani P, Baldelli AM, et al. Neuroprotective Effect of Reduced Glutathione on Oxaliplatin-Based Chemotherapy in Advanced Colorectal Cancer: A Randomized, Double-Blind, Placebo-Controlled Trial. J Clin Oncol. 2002;20(16):3478-83.

225. Messori L, Merlino A. Cisplatin binding to proteins: A structural perspective. Coord Chem Rev. 2016;315:67-89.